ACIDS

40

STRUCTURE
BIOSYNTHESIS AND
FUNCTION

NUCLEIC ACIDS

Some of the most spectacular and significant advances in modern biology, perhaps in modern science, in recent years, have been concerned with researches on nucleic acids, an important group of biological polymers of ubiquitous occurrence. A great deal of effort has been put in the last fifteen years in the direction of understanding the structure, the mechanism of biosynthesis and the function of the two types of nucleic acids, deoxyribonucleic acid and ribonucleic acid. This publication is a compilation of papers presented at an international symposium on the above aspects of nucleic acids held at the Regional Research Laboratory, Hyderabad, India, from 16th to 22nd January, 1964, under the auspices of the Council of Scientific & Industrial Research, Ministry of Education, Govt. of India, New Delhi.

The symposium was attended by 33 invited participants from U.S.A., U.K., France, Germany, Israel, Japan and India, and nearly 100 observers from some 50 institutions in India. The primary objectives of the symposium were to provide an opportunity for a close and informal contact between workers on nucleic acids in India and abroad; to take stock of the work being done in this field in India in the context of the extensive investigations being carried out elsewhere; and to emphasize that this field, like other important areas of research in modern biology, provides a meeting ground for many, varied disciplines. The papers presented at the symposium covered many diverse aspects of structure, biosynthesis and function of nucleic acids.

The publication should be of interest to all who have been engaged in research on nucleic acids from any point of view.

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NUCLEIC ACIDS

STRUCTURE, BIOSYNTHESIS AND FUNCTION

Symposium Organized by

The Regional Research Laboratory, Hyderabad
January 16-22, 1964

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Preface

This publication is a compilation of papers presented at a Symposium on Nucleic Acids held at the Regional Research Laboratory, Hyderabad, India, from January 16-22, 1964, under the auspices of the Council of Scientific & Industrial Research, Ministry of Education, Government of India, New Delhi. Some of the most spectacular and significant advances in modern biology, perhaps in modern science, in recent years, have been concerned with researches on nucleic acids, an important group of biological polymers of ubiquitous occurrence. A great deal of effort has been put in the last fifteen years in the direction of understanding the structure, the mechanism of biosynthesis and the function of the two types of nucleic acids, the deoxyribonucleic acid (DNA) and the ribonucleic acid (RNA).

The structure of DNA originally proposed by J. D. Watson and F.H.C. Crick in 1953 is now unequivocally established. It is shown to be an unbranched molecule in which two polynucleotide strands are held together by hydrogen bonds. Any one strand in a particular DNA molecule contains a specific sequence of the four constituent bases; this sequence characterizes the DNA molecule and also determines the sequence of bases in the other strand of the DNA molecule. At least the salient features of the mechanism of synthesis of DNA are now clear; in the presence of the enzyme DNA polymerase (first described by A. Kornberg in 1958) and the four deoxyribonucleoside triphosphates, DNA can act as a template for its own replication by a semi-conservative mechanism, the two daughter molecules inheriting one each of the parent strands. However, unequivocal demonstration of biological activity in DNA synthesized *in vitro* by the Kornberg system, has proved to be an unexpectedly difficult task.

The broad function of DNA is also now more or less fully understood. There is no doubt that this is the genetic material responsible for the remarkable and important phenomena of "transformation", first described by O. T. Avery in 1944, and "transduction", described some ten years later by J. Lederberg, and that, if not the only, it is the predominant self-replicating material in biological systems. In the case of lower organisms like bacteria, the entire single chromosome has been shown to consist of a single molecule of DNA. Portions of a DNA molecule correspond to different genes, and considerable success has been achieved, e.g. by S. Benzer, in the last ten years, in determining the sequence of genes in a DNA molecule;

there is, however, scope for even "finer" genetic analysis than has been so far conducted.

DNA manifests its genetic function primarily by carrying the information for determining the amino acid sequence in the various proteins of the cell. The code for the determination of this sequence is represented by non-overlapping nucleotide triplets in the DNA molecule; the code is degenerate, more than one triplet representing a single amino acid. Majority (but not all) of the triplets have now been decoded. This recent, dramatic discovery, which followed investigations conducted at a very rapid pace in the last four years, primarily by three groups of workers led by M. W. Nirenberg, S. Ochoa and F.H.C. Crick, constitutes a major triumph of modern biology.

DNA is also involved in regulatory mechanisms which determine *when* an enzyme should be synthesized. The presence of genes which are involved *not* in the determination of the sequence of amino acids in an enzyme protein but in the regulation of its synthesis has been demonstrated unequivocally, following the pioneering work of J. Monod and his colleagues during the last five years; the nature of the "repressor", the regulatory substance the synthesis of which is under genetic control, is, however, not yet established. Although, it seems probable that DNA is a direct or indirect determinant of morphogenesis, its exact role in this important phenomenon, which has been very much talked about but very little understood, is not clear.

It has been convincingly demonstrated in the last five years following a prediction by F. Jacob and J. Monod and the pioneering work of J. D. Watson, F. Gros and S. Brenner, that DNA transmits its code to protein through a RNA molecule (termed the "messenger" RNA) which is formed with the DNA serving as a template. From experiments on the replication of the bacteriophage ϕX—174, which contains a single-stranded DNA (one of the few known exceptions to the two-strand rule), evidence has been obtained very recently by S. Spiegelman which seems to suggest that only one of the two strands of DNA may be involved in the formation of the messenger RNA active in protein synthesis; confirmation of this observation using other systems will be awaited with great interest.

Two other types of RNAs which are also involved in protein synthesis are known: the small molecular weight "soluble RNA" and the high molecular weight "ribosomal RNA", the latter is a part of the ribonucleoprotein particles (ribosomes) which are the site of protein synthesis. It is certain that the amino acids are first attached to "soluble RNA" (s-RNA, transfer RNA) molecules (there being at least one different s-RNA for each amino acid), and then brought to the messenger RNA for formation of the polypeptide chain. The sequence in which the amino acids are joined together is determined by the sequence of the nucleotides in the messenger RNA; the messenger has been shown to recognize the amino acids through the s-RNAs to which they are attached. Although the mechanisms are not fully established, messenger RNA appears to serve as a 'tape' which holds together several ribosomes, which, in turn, appear to be responsible for keeping the messenger in the proper configuration which would enable it to be effective in assembling the sequence of amino acids for which it has brought the information from DNA. The precise role of ribosomal RNA in protein synthesis is also not clear.

The mechanism of the synthesis of messenger RNA is fairly well understood; its formation needs an enzyme (RNA polymerase), first described by S. Weiss in 1959, DNA (as is to be expected) and the four ribonucleoside triphosphates. On the other hand, no definite information is available on the mechanisms of syntheses of soluble and ribosomal RNAs.

Out of the three above mentioned types of RNA, significant information about structure is available only in the case of s-RNA. s-RNA appears to exist as a single, unbranched polyribonucleotide chain folded over itself to give a predominantly double-helical structure (M.H.F. Wilkins, 1961) in which in the case of every s-RNA molecule capable of accepting an amino acid (irrespective of the amino acid for which the s-RNA may be specific), the sequence of bases in the terminal trinucleotide is -cytosine-cytosine-adenine; the amino acid is hooked to the terminal adenine through an ester linkage. However, leave aside the primary structure (the elucidation of which has to be preceded by the development of a method for obtaining a preparation of s-RNA which is specific for only one amino acid and, therefore, likely to represent a single molecular species), the nature and location of even the sites on s-RNA molecules which determine their specificities towards the amino acid on one side and the messenger RNA on the other, are far from clear.

In addition to the above three kinds of RNA, recently the presence of homopolymers, like polyadenylic acid which was first synthesized *in vitro* by means of the enzyme, polynucleotide phosphorylase by S. Ochoa and his coworkers in 1955, has been demonstrated in some biological systems. The exact biological function of these homopolymers is not known, although it seems to be fairly clear how they are synthesized.

It has been shown recently that RNA-containing viruses can induce the formation of an enzyme, RNA synthetase, which can replicate the viral RNA. This seems to be the only other self-replicating system known besides DNA, although the existence of other self-replicating systems involving nucleic acids cannot by any means be ruled out on the basis of existing knowledge.

This description of some of the salient features of investigations on nucleic acids in the last decade or so will, it is hoped, show even to a lay reader, that nucleic acids are intimately involved in two extremely important biological phenomena which indeed are the hall-mark of life, i.e. heredity and enzyme synthesis. It will also be seen that while tremendous strides have been taken in understanding the nature and the mechanism of this involvement, making the chemistry, biochemistry and physiology of nucleic acids as perhaps one of the most exciting, thought-provoking and intellectually stimulating areas in modern biology, there are many aspects which are not clearly understood. The Symposium, part of the proceedings of which are incorporated in this publication, was organized as a broad-based meeting for discussion of studies on structure, biosynthesis and function of both types of nucleic acids. Although no major new discoveries were announced (in these days of rapid communication of ideas and results, particularly in biology, it is rare for major discoveries to be announced for the first time at meetings of this kind), light was thrown on many important aspects of chemistry and biochemistry of nucleic acids. Thirty-three papers were presented by 21 invited participants from outside India and 13 from India; out of these, 31 papers are included in this publication. The Symposium was, in addition, attended by approximately 100 observers from some 50 institutions in India.

The Symposium, from the point of view of its organization, content and participation, was the first of its kind to be arranged in the country. There was no formal inauguration of the Symposium. The admission at the meetings was restricted to the invited participants and observers. The meetings were held under an informal, unrestrained and academic atmosphere, and the keynote of the Symposium week was the earnestness and seriousness of purpose, delightfully tempered with wit and humour which provided some compensation for the physical endurance required in sitting (even on cushioned chairs) for six hours a day for six days. An effort was made to provide as much time as possible, in between and after the Symposium sessions, for discussion; during the sessions, 50 minutes were available for the presentation and discussion of each paper. One whole day was devoted to an excursion to places of interest in and near Hyderabad.

A significant feature of the Symposium week were three popular lectures (not reproduced in this publication) on "Structure and Function of Deoxyribonucleic Acid—the Genetic Material", "Viruses and Molecular Biology", and "Nucleic Acids and Cancer Chemotherapy", given on three evenings by Dr F.H.C. Crick, Prof. G. Schramm and Dr C. Heidelberger, respectively. These lectures were held under the joint auspices of the Regional Research Laboratory, Hyderabad Science Association, the Hyderabad Branch of the Indian Medical Association and the Andhra Pradesh Akademi of Sciences, at Ravindra Bharati, the leading theatre hall of Hyderabad, and were delivered to a packed audience of nearly 1200 at each lecture. We, and we hope the speakers too, were gratified to see such a response from the people of Hyderabad, where, as perhaps everywhere else in the country molecular biology, DNA and RNA, are words not frequently heard even among scientists, or seen in print even in the scientific journals of the country !

We had the following objectives in mind in arranging this meeting:

(a) to provide an opportunity for a close, informal contact between active workers in the field of nucleic acids in the country, other workers in the country who are directly or indirectly interested in the field of nucleic acids, and a limited number of prominent workers in this area from outside India;

(b) to create an awareness in the country of the great importance of nucleic acids in the spectrum of modern biology;

(c) to create an awareness in the country of the fact that the significant contributions in this field have been made by workers who have been trained in varying disciplines of science, and that this field, like the other important areas of research in modern biology, provides a meeting ground for various disciplines; and

(d) to stimulate research on nucleic acids in the country.

The organizers do have the feeling that the first three objectives were satisfactorily achieved. To what extent the last objective had been achieved, only time will tell.

So much about the nature, objectives and the results of the Symposium. Now a few words about this publication.

We had originally planned to publish the entire proceedings of the Symposium, including all the papers presented, and the complete discussions. However, this publication now contains 31 out of the 33 papers presented at the Symposium and does not incorporate any discussion. The tape recordings of the discussions left much to be desired. Further, we felt that the draft of the transcribed discussion, if published, should be approved by the concerned participants, and this would have taken much time of the participants. We, however, hope that in spite of these omissions, this collection of papers will be of use to workers in the field, if only for the reason that the papers cover a very wide range.

Much of the credit for whatever success the meeting achieved goes to Dr S. Husain Zaheer, Director-General, Scientific & Industrial Research, and Ex-officio Secretary, Ministry of Education, Government of India, New Delhi, and Dr G. S. Sidhu, Director of this Laboratory, without whose continuous interest and support it would not have been possible to arrange this meeting. I should also like to take this opportunity to thank, on behalf of the organizers of the Symposium, the various organizations outside India, particularly the Wellcome Trust, London, which made a special grant direct to us, who provided funds to meet the travel expenses of invitees from outside India. Travel to India from most parts of the world is an expensive business and we were gratified to see that so many organizations found it worthwhile to make it possible for eminent scientists from their (and other) countries to attend the Symposium. I am also personally indebted to a very large number of colleagues in the Laboratory, both from the Biochemistry Division and from the other divisions, for taking meticulous care of the various aspects of the organization of this Symposium.

There has been an inordinate delay in bringing out this publication. It was our hope that the publication would be out within 6 months from the Symposium. However, it has taken more than twice this amount of time. This has been primarily due to the fact that some of the manuscripts were received from the authors as late as September 1964. It is wellknown that biochemists in general and molecular biologists in particular are pretty averse to writing papers for symposia and, as such, we expect that readers would understand the delay in bringing out this publication, and find it of some use even though it may be somewhat out of date by the time it appears. This would be particularly true of those papers which were submitted by conscience-stricken authors just before or after the Symposium. The blame for any out-dated information contained in the publication should, therefore, be put on us and not on the authors.

P. M. Bhargava

Hyderabad
June 10, 1964

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On the Natural Occurrence of Polyadenylate and its Synthesis from Adenosine Triphosphate*

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Rapid advances have been made in the past few years in elucidating the enzymic pathway for the synthesis of ribonucleic acid with deoxyribonucleic acid as a template (for review see Ref. 1). The question of whether all of the RNA species found in the cell are formed in this fashion has been raised, and based upon the observation that all RNA synthesis can be prevented by high doses of actinomycin D, the conclusion has been drawn that all RNA is indeed synthesized on a DNA template[2,4]. However, one cannot eliminate the possibility that cytotoxic agents such as actinomycin D when used in high concentrations may have secondary effects upon the cell in addition to the block of RNA synthesis on a DNA template.

The possibility that other RNA synthetase systems exist in cells (excluding RNA-virus infected cells) is suggested by the sizeable number of crude or partially purified enzymes that incorporate nucleoside triphosphates into polynucleotides without the direct involvement of DNA. Although an RNA requirement is often encountered, the product formed has not as a rule had an obvious compositional relationship to the primer used. A unique aspect of some of these preparations is the formation of homopolymeric chains from nucleoside triphosphates, usually either adenosine triphosphate or uridine triphosphate. Enzymes that have been reported to carry out polyadenylate and polyuridylate synthesis are summarized in Tables 1 and 2. Chain lengths of the homopolymeric products vary from several to several hundred, and in a number of instances evidence

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*This investigation was supported by research grants from the American Cancer Society, the U.S. Public Health Service (No. AM 07872-01 BIO), and the U.S. Atomic Energy Commission Contract AT (30-1)-1818.

TABLE 1—Synthesis of Polyadenylate Sequences

Enzyme source	Primer	Cofactors	Ref.
Mammals and birds-liver chromatin	DNA	Mg^{2+} + nicotinamide Ribotide	5
Esch. coli-RNA polymerase	DNA	Mg^{2+}	6, 7
Ascites nuclei-ribosomes	Ribosomal RNA	Mg^{2+}	8
Esch. coli-supernatant fraction	Ribonucleotidyl-DNA	Mg^{2+} (Mn^{2+})	9
Esch. coli-ribosomes	Ribosomal RNA	Mg^{2+}	10
Rat liver-supernatant fraction	Ribosomal RNA (Polyadenylate + polyuridylate) Polycytidylate Polyuridylate	Mn^{2+}	11
Chick embryo-chorioallantoic membrane	Ribosomal RNA (Polyadenylate + polyuridylate) Polyadenylate Polyuridylate	Mn^{2+}	12
Thymus nuclei	Polyadenylate	Mg^{2+}	13, 14
Thymus nuclei	Any RNA Polyadenylate	Mn^{2+}	(This report)

TABLE 2—Synthesis of Polyuridylate Sequences

Enzyme source	Primer	Cofactors	Ref.
Ascites nuclei-chromatin	DNA	Mg^{2+} + Mn^{2+}	15
Esch. coli-RNA polymerase	DNA	Mg^{2+}	7
Ascites nuclei-ribosomes	Ribosomal RNA Polyuridylate	Mg^{2+}	8
Rat livers-upernatant fraction	Ribosomal RNA Polyuridylate Polycytidylate Ribonucleotidyl-DNA	Mg^{2+}	16
Pigeon liver-microsomes	Ribosomal RNA	Mg^{2+} or Mn^{2+}	17

is available suggesting that the product is built up as a terminal sequence on the added polynucleotide primer.

Thus, the possibility that long homopolymeric sequences may be natural products is based upon the existence of enzymes for their synthesis. It is not in general known whether such homopolymers are artefacts created *in vitro* or whether they exist normally within the cell as components of the heterogeneous RNA population. To throw some light on this question, and to emphasize, by example, the possible diversity of polyribonucleotide synthetase systems, this communication will review two enzymic systems found in extracts of calf thymus nuclei that are concerned with polyadenylate synthesis. This work began a number of years ago with the observation that soluble extracts of Ehrlich ascites and calf thymus nuclei incorporated adenosine triphosphate into non-terminal positions in polynucleotide. Upon purification, two enzymic activities were separated—one specific for adenosine triphosphate and one for cytidine triphosphate (Table 3). The following discussion refers to the polyadenylate synthesizing system.

Nature of the product

With ^{32}P-labelled adenosine triphosphate it was evident, as shown in Table 4, that each incorporated adenylate unit occurred next to another adenylate unit. Thus, the enzyme catalyzed the synthesis of polyadenylate. Other reactions occurred in the crude extract.

TABLE 3—SUBSTRATE SPECIFICITY OF NUCLEOSIDE TRIPHOSPHATE POLYMERASES ISOLATED FROM THYMUS NUCLEI

Labelled substrate	Adenosine triphosphate polymerase mμmoles/hr/mg. protein	Cytidine triphosphate polymerase mμmoles/hr/mg. protein
ATP	62·0	1·5
CTP	0·1	21·0
UTP	0	0
GTP	0	0

TABLE 4—NEAREST NEIGHBOR RELATIONSHIP AFTER INCORPORATION OF α-^{32}P-ADENOSINE TRIPHOSPHATE

Dinucleotide sequences	Relative frequency	
	Crude extract	Purified enzyme
ApA	0·42	0·99
CpA	0·36	0·01
GpA	0·20	0
UpA	0·02	0

Nature of the primer

The requirements for polyadenylate synthesis were enzyme, adenosine triphosphate and Mg^{2+}. However, the purified enzyme contained 2% RNA, and it was possible to show that this component was intimately involved in the reaction. RNA free of protein was obtained by heat denaturation; and protein relatively free of polynucleotide, by treatment with an RNase from squid cecal fluid[18]. Both the protein and polynucleotide components were required for activity.

As shown in Table 5, the isolated polynucleotide primer was inactivated by treatment with some nucleases such as the squid RNase but not by others. It was evident that ability to split bonds next to adenylate units was a prerequisite for primer inactivation, suggesting that the actual priming component was polyadenylate itself. This was confirmed by passing the crude primer through a column of chemically synthesized polythymidylate covalently bound to cellulose[19,20]. At high ionic strengths polyadenylate was expected to form a hydrogen bonded complex with the polythymidylate and thereby adhere to the cellulose. Lowering the ionic strength should result in dissociation of the complex with subsequent elution of the adsorbed polyadenylate. The results of such an experiment are shown in Fig. 1. Priming activity was detected only in the fraction B that was adsorbed at high ionic strength and eluted at low ionic strength, and the only nucleotide released upon alkaline hydrolysis of this fraction was adenylate. Approximately 10% of the crude primer and 0·25% of the total nuclear RNA was in fact polyadenylate. The thymus polyadenylate isolated in this way was a highly effective primer resulting in net synthesis of polyadenylate (Fig. 2).

TABLE 5—EFFECTS OF NUCLEASES ON THE ACTIVITY OF PRIMER ISOLATED FROM ADENOSINE TRIPHOSPHATE POLYMERASE

Nuclease	Inactivation of primer*	Hydrolysis of adenylate bonds
Pancreatic RNase	0	0
Squid RNase	+	+
Takadiastase RNase I	0	0
Takadiastase RNase II	+	+
A. agilis RNase	+	+
Venom diesterase	+	+
Pancreatic DNase	0	0

*Inactivation represents destruction of at least 75% of the priming activity.

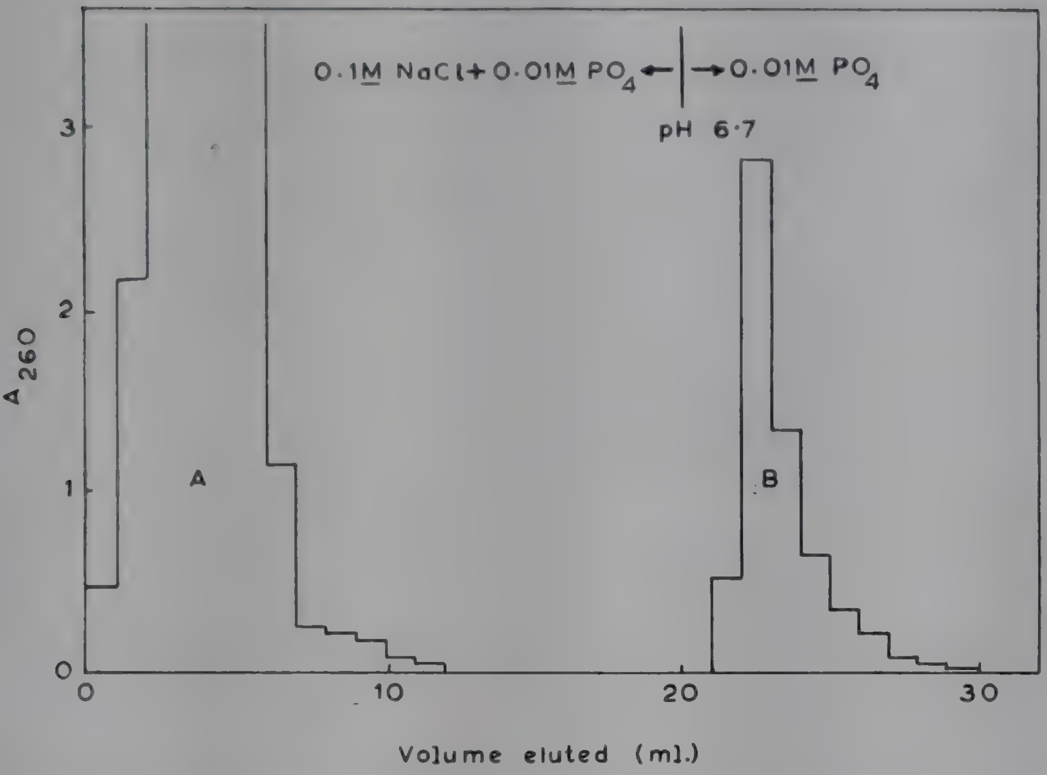

FIG. 1—FRACTIONATION OF THE POLYNUCLEOTIDE PRIMER ON POLYTHYMIDYLATE CELLULOSE: Approximately 2·5 mg. of crude primer in 6·5 ml. of the indicated saline-phosphate were washed through a column 2·0 cm. long by 1·0 cm. in diameter. The retained polynucleotide (10% of the total) was eluted with 0·01 *M* phosphate

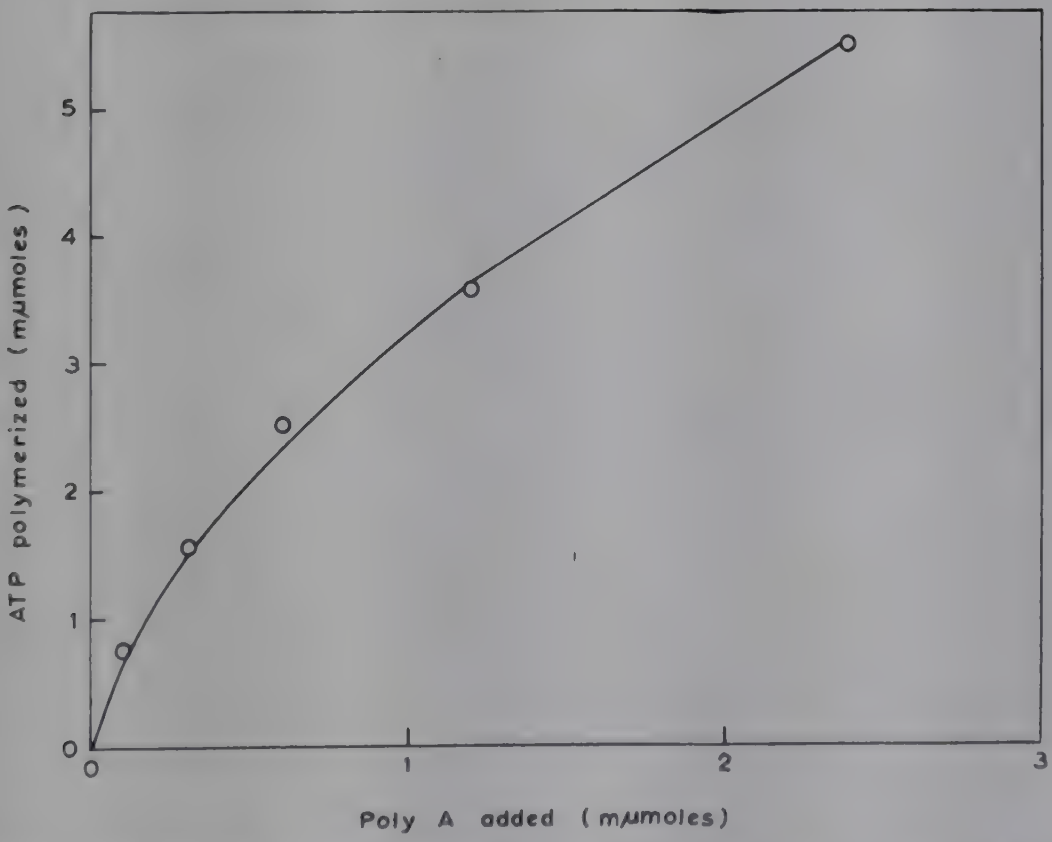

FIG. 2—NET SYNTHESIS OF POLYADENYLATE WITH THYMUS POLYADENYLATE AS PRIMER: Primer (shown as adenylate residues) was added to .1 mg. of polynucleotide-depleted enzyme and incubated with adenosine triphosphate for 30 min. at 37°C.

TABLE 6—PRIMING ACTIVITY OF SYNTHETIC HOMOPOLYMERS

Added polymer	Reactivation of apoenzyme %	Inhibition of intact enzyme %
Polyadenylate	65	30
Polyinosinate	15	20
Polycytidylate	6	0
Polyuridylate	0	75

A number of different cytoplasmic and nuclear RNA fractions, as well as DNA, have been found inactive as primers. Results with synthetic polynucleotides prepared with polynucleotide phosphorylase are shown in Table 6. Of these homopolymers polyadenylate was an effective primer and polyinosinate had some activity. The others were inactive. For reasons not clearly understood excess polyadenylate added to the intact enzyme resulted in some inhibition. Polyuridylate was observed to be a strong inhibitor, and it was possible to titrate the enzyme with polyuridylate, complete inhibition being observed when the amount of added polyuridylate was approximately equal to the normal content of polyadenylate in the enzyme preparation.

In contrast to the high molecular weight of polyadenylate prepared with polynucleotide phosphorylase, native thymus polyadenylate as isolated was more comparable in size to soluble RNA. This is indicated in the distribution pattern obtained from a methyl albumin column as shown in Fig. 3. High molecular weight polyadenylate was retained strongly, while both thymus polyadenylate and the product of the adenosine triphosphate polymerase reaction were eluted in the range of NaCl concentrations characteristic of soluble RNA. The elution profile from polythymidylate cellulose columns also indicated that thymus polyadenylate was smaller and more hetero-disperse than the polyadenylate made with polynucleotide phosphorylase (Fig. 4).

Priming mechanism

In any primed polynucleotide synthesis one is faced with the question, does the primer function as a nucleus with the incorporated units added to pre-existing chains or is it a true template that directs the synthesis of new copies of itself. With most of the RNA-requiring polyadenylate and polyuridylate synthesizing systems listed in Tables 1 and 2 there is little question that the homopolymer grows as a chain extension on the primer. While the difficulties associated with complete removal of a nuclear RNase make a decision difficult, there are a number of arguments for a template role for polyadenylate in the thymus system described here.

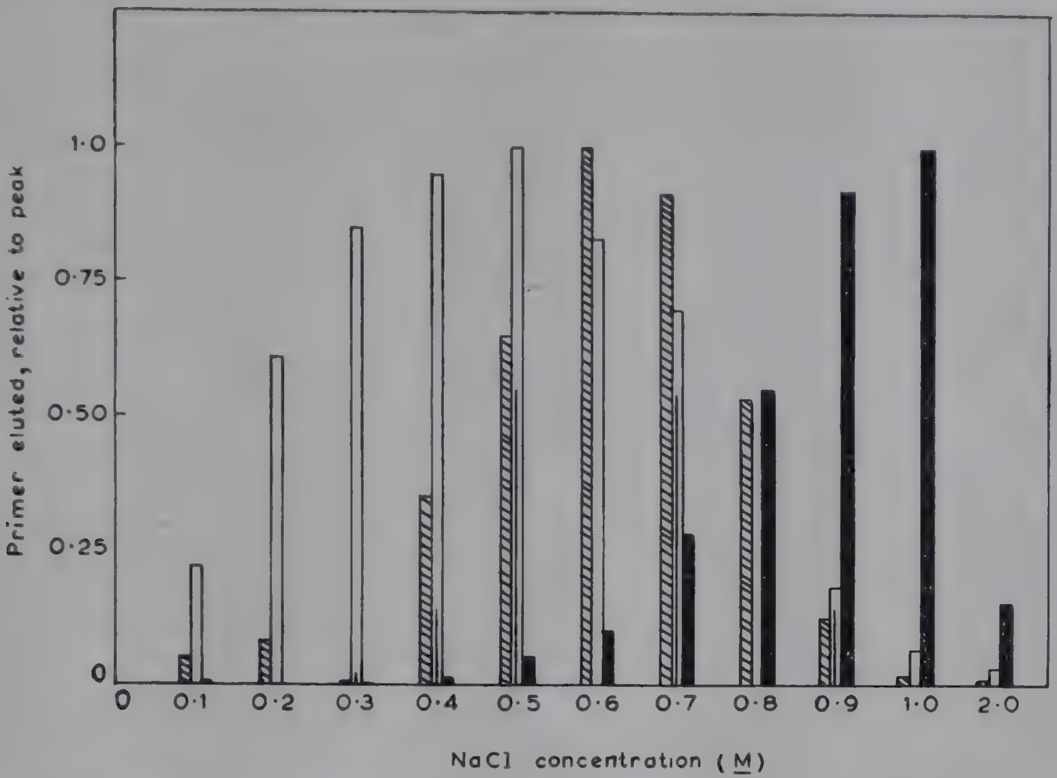

FIG. 3—FRACTIONATION OF PRIMER AND REACTION PRODUCT ON A METHYL ALBUMIN COLUMN: Column preparation and stepwise elution were carried out as described by Sueoka[21]. The open bars represent the elution profile of crude, unfractionated primer. Some fractions were assayed for priming activity (i.e. polyadenylate content) and this is represented by the height of the line within the bar. Labelled product of the polymerization reaction is shown by the shaded bars. For comparison, the elution of high molecular weight polyadenylate made with polynucleotide phosphorylase is indicated by the black bars

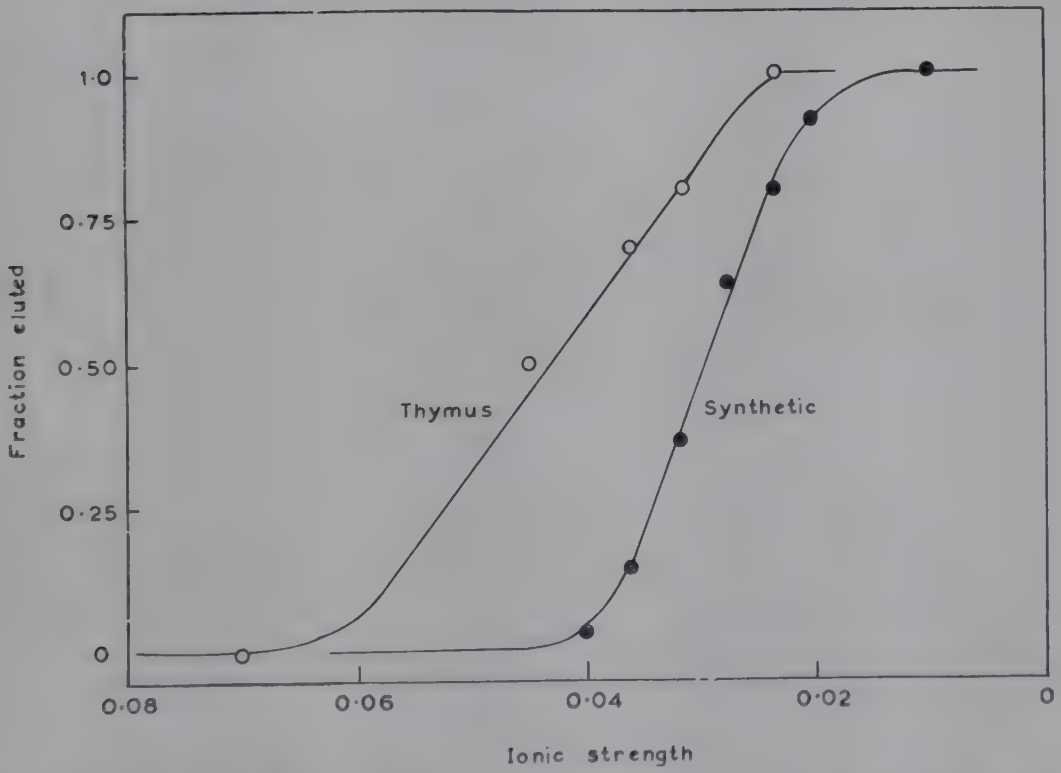

FIG. 4—ELUTION PROFILES OF THYMUS POLYADENYLATE AND POLYADENYLATE MADE WITH POLYNUCLEOTIDE PHOSPHORYLASE FROM A POLYTHYMIDYLATE CELLULOSE COLUMN: Samples were adsorbed on a 2·0 by 1·0 cm. column in 1·0 M NaCl and eluted stepwise at decreasing ionic strengths

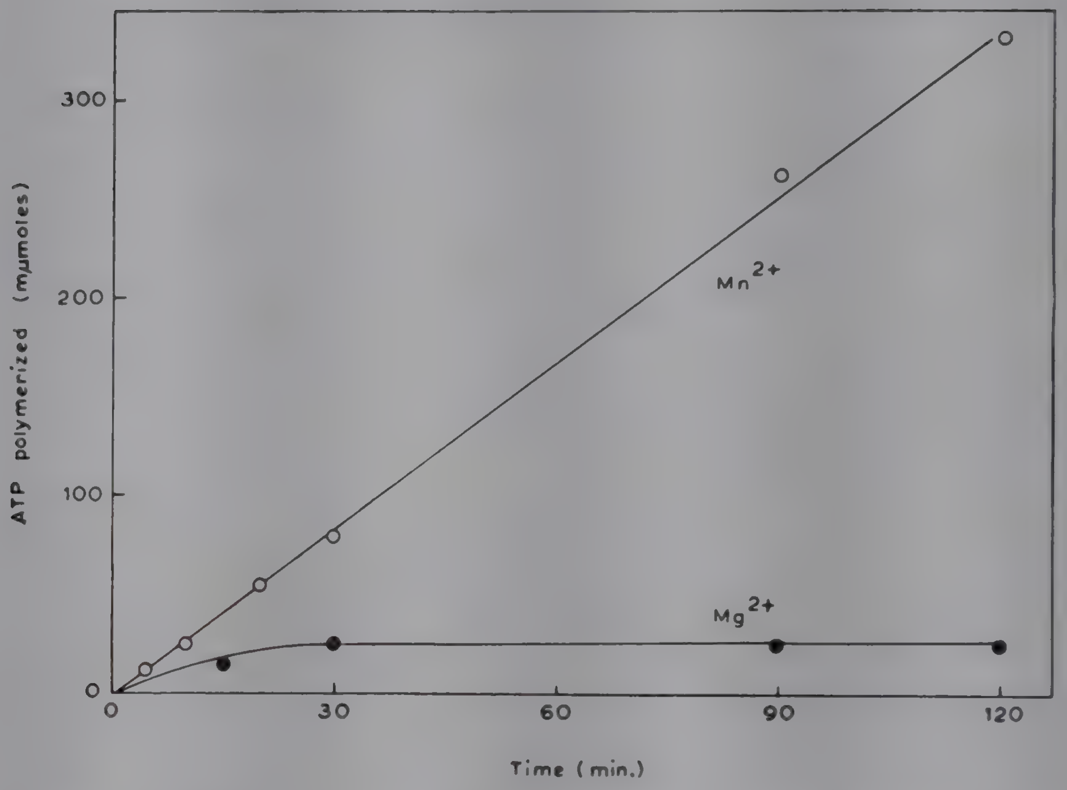

Fig. 5—Effect of substituting Mn^{2+} for Mg^{2+} on the rate of polymerization of polyadenylate

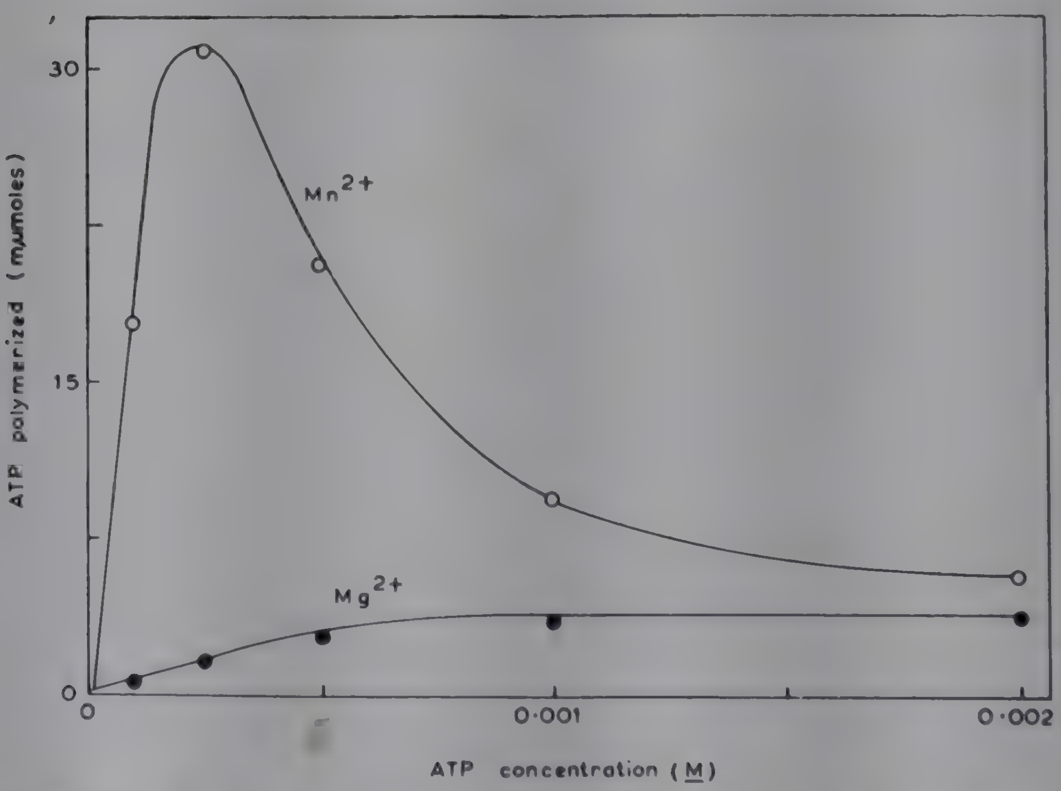

Fig. 6—Effect of adenosine triphosphate concentration on the activity of the polymerase: Divalent cation concentration was maintained at twice the adenosine triphosphate concentration

Prime specificity. The high degree of specificity for a particular primer is not shown by those enzymes known to utilize a chain elongation mechanism.

Observable increases in chain length. Where observations have been made, homopolymers which grow by addition to a primer show an increase in chain length with time. This seems, in fact, to be true of two other polymerases we have examined in thymus extracts. With adenosine triphosphate polymerase, however, chain length was independent of time, the length of the product at the beginning of the reaction being indistinguishable from that present an hour later.

Ineffectiveness of oligonucleotide primers. It has been suggested that polynucleotide phosphorylase functions by adding mononucleotide units to primer chains, and this has been unequivocally demonstrated with small oligonucleotides as primers[22]. When these oligonucleotides (obtained from Dr. Leon Heppel) were tested for priming activity with the thymus polymerase, the results shown in Table 7 were obtained. Diadenylate and triadenylate were devoid of priming activity. With pentanucleotide as a primer a single terminal group was added but no evidence for chain growth was obtained. Thus oligonucleotides were incapable of priming polyadenylate synthesis.

Equivalence of end groups. If the incorporated adenylate units were added to a pre-existing polyadenylate chain, adenosine would be sole radioactive end. In fact, the amounts of nucleoside and 5′-phosphate ends were comparable as shown in Table 8.

Ineffectiveness of blocking the 3′-hydroxyl group. Periodate oxidation was used to remove a terminal adenosine from polyadenylate leaving the molecule with a phosphate on the 3′-hydroxyl group[23]. The data in Table 9 indicate that this treatment had no effect on the priming activity suggesting that a free 3′-hydroxyl end was not required for priming.

While all these results indicate that polyadenylate functions as a template for its own synthesis, it can be argued that this evidence loses its cogency if only a few RNase hits occur. An internal control for this possibility is provided by the fact that the same purified enzyme preparation can be shown to contain another type of adenosine triphosphate polymerase where polyadenylate synthesis is in fact the result of a chain extension process.

Mn^{2+}-activated adenosine triphosphate polymerase

An attempt was made to substitute Mn^{2+} for Mg^{2+} in the hope that the polymerase but not the nuclease would function with Mn^{2+}. As shown in Table 10, the nuclease was in fact active only in the presence of Mg^{2+} and an equivalent amount of Mn^{2+} added to the Mg^{2+}-activated system resulted in complete inhibition. Polymerase activity, on the other hand, was greatly enhanced by Mn^{2+}. The reaction began at a faster rate and continued in a linear fashion for a longer time as shown in Fig. 5.

TABLE 7—ADENYLATE OLIGONUCLEOTIDES AS PRIMERS FOR THE POLYMERASE

[Priming activity was assayed with 1 mg. of primer-depleted enzyme using equivalent amounts of each of the primers. Activities are expressed relative to the native primer taken as 100]

Primer added	Relative utilization of adenosine triphosphate	Location of incorporated adenylate % terminal
Thymus polyadenylate	100	2
Synthetic polyadenylate	64	2
Diadenylate 5′-phosphate	0	
Triadenylate 5′-phosphate	0	
Tetraadenylate 5′-phosphate	10	
Pentaadenylate 5′-phosphate	22	98

TABLE 8—END GROUPS OF ^{14}C-POLYADENYLATE PREPARED WITH ADENOSINE TRIPHOSPHATE POLYMERASE

[The reaction product was hydrolyzed for 18 hr in 0·3 *M* KOH at 37°C. after which the neutralized hydrolysate was chromatographed on Dowex-l-acetate]

Fractions isolated after alkaline hydrolysis	Relative ^{14}C content
Adenosine	2·1
2′- and 3′-adenylate	100
2′, 5′- and 3′, 5′-adenylate	1·4

TABLE 9—EFFECT OF 3′-PHOSPHATE ON PRIMER ACTIVITY

Added primer	Relative adenosine triphosphate incorporation
Endogenous	100
None	4·2
Polyadenylate*	32
Polyadenylate-3′-phosphate	29

*High molecular weight polyadenylate (polynucleotide phosphorylase). The terminal adenosine was removed from a sample of this polyadenylate to give a terminal 3′-phosphate in the primer designated as polyadenylate-3′-phosphate in the table.

However, present evidence indicates that the Mn^{2+} polymerization is not the same reaction as the one observed with Mg^{2+}. Despite the inhibition of the nuclease, chain lengths of polyadenylate formed with Mn^{2+} were shorter (20 units) than those formed with Mg^{2+}. The results plotted in Fig. 6 indicate that enzyme saturation occurred at a lower concentration of ATP in the Mn^{2+} reaction. While the Mg^{2+} reaction was unaffected by excess adenosine triphosphate, the results with Mn^{2+} suggest that two adenosine triphosphates reacted to form an inactive complex with the enzyme. Most significantly, there was a striking loss of primer specificity when Mn^{2+} was substituted for Mg^{2+} (Table 11).

In contrast to the Mg^{2+}-activated reaction, but like the other polymerases characterized by low primer specificity, the data summarized in Fig. 7 show that chain lengthening occurred during the Mn^{2+} reaction.

TABLE 10—CATION REQUIREMENT OF NUCLEAR RNASE

[Quantities used in a 1 ml. reaction volume were 0·20 mg. polyadenylate, 0·20 mg. enzyme, 1 μmole $MgCl_2$ and 1 μmole $MnCl_2$]

Additions				Relative degradation of polyadenylate
Polyadenylate	Enzyme	Mg^{2+}	Mn^{2+}	
+	−	−	−	0·7
+	+	−	−	5
+	+	+	−	100
+	+	−	+	12
+	+	+	+	12

TABLE 11—PRIMER SPECIFICITY FOR Mn^{2+} AND Mg^{2+} ACTIVATED ADENOSINE TRIPHOSPHATE POLYMERASES

[Priming activities are expressed relative to primer obtained from the purified polymerase]

Primer	Relative activity	
	Mg^{2+}	Mn^{2+}
Crude ATP primer	100	4000
Crude CTP primer	6	8700
Yeast sRNA	14	4600
Polyadenylate*	60	6900
Polyuridylate*	0	700

*These homopolymers were synthetic preparations (polynucleotide phosphorylase).

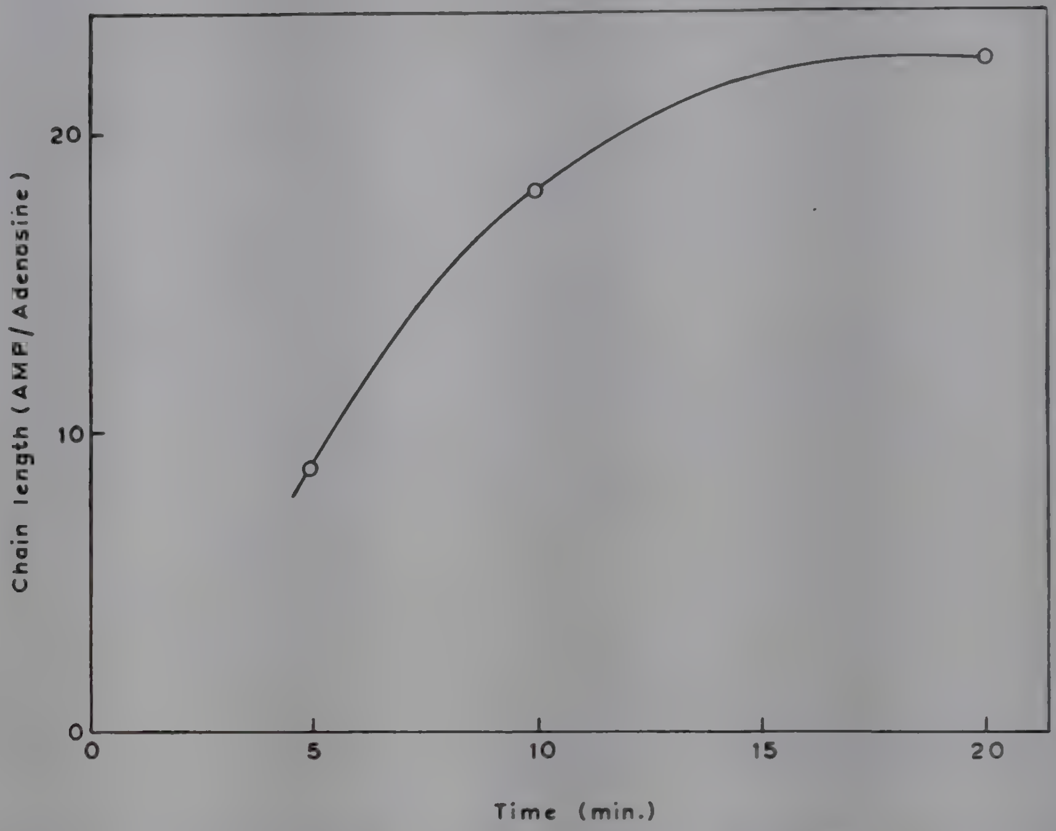

FIG. 7—EFFECT OF INCUBATION TIME ON CHAIN LENGTH WITH THE Mn^{2+} ACTIVATED ENZYME: Samples were hydrolyzed with alkali at the times indicated and the relative proportions of internal and terminal adenylates estimated after separation of adenosine and 2′- and 3′-adenylate on Dowex-1-acetate columns

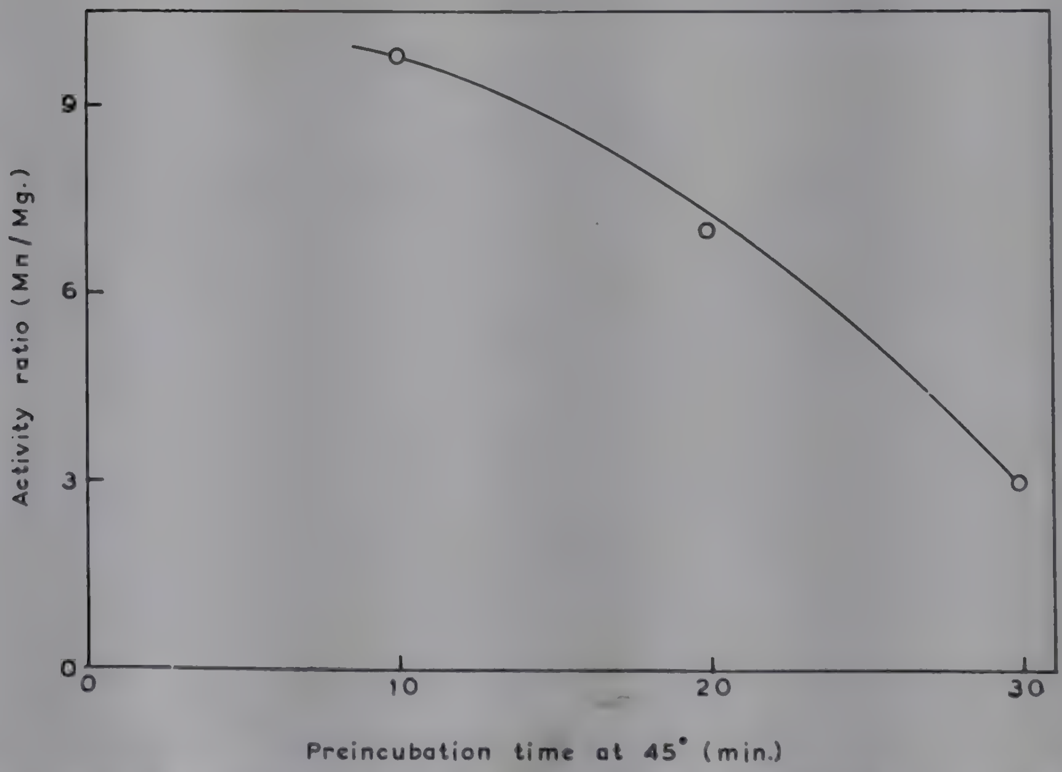

FIG. 8—THERMAL INACTIVATION OF THE Mg^{2+} AND Mn^{2+} POLYMERASE ACTIVITIES: Data are expressed as the ratio of activity with Mn^{2+} to activity with Mg^{2+} after the indicated times of preincubation at 45°C.

The wide disparity of reaction characteristics between the Mg^{2+} and Mn^{2+}-activated systems suggested that two separate enzymes were involved. This conclusion has been augmented by the fact that it has been possible to obtain the Mn^{2+}-activated preparation free of the Mg^{2+} activity, and as shown in Fig. 8, the two activities are inactivated by heat at different rates. The Mn^{2+}-activated system is similar to that described in rat liver recently by Klemperer[11].

The fact that the Mn^{2+} enzyme shows all of the properties characteristic of chain elongation on the added primer while the Mg^{2+} enzyme shows none of these properties, strengthens the conclusion that polyadenylate acts as a template in the latter system for its own replication. In homopolymer synthesis, as in this case, one does not have the constraints which limit one to the adenylate-thymidylate (or uridylate) pairs as in DNA synthesis. Hydrogen bonding could function as a directing influence for adenine-adenine interaction involving the amino group and N-7 as in the known double-stranded helix that has been observed with polyadenylate[24]. Nor can one *a priori* rule out N-1 as an alternative hydrogen bonding site.

The evidence presented here has indicated that homopolymer synthesis can be a fairly specific reaction and that at least one homopolymer, polyadenylate, does in fact occur in nature. Polyadenylate is not unique to calf thymus nuclei. We have found it in commercial yeast RNA (0·1%) and even more in yeast soluble RNA prepared by the method of Monier *et al.*[25] where 0·25% of the RNA is polyadenylate. Preliminary results obtained with rat liver by Bruce Nicholson suggest that polyadenylate is present in liver and that polyadenylate synthesis can occur *in vivo*.

The fact that at least one homopolynucleotide, polyadenylate, occurs in nature in association with an enzyme specific for its own synthesis suggests that at least some of these step children of the RNA family have biological functions which are presently unknown. We have at present no definite evidence for any specific function for polyadenylate, but several possibilities that are under consideration might be mentioned. (*a*) Polyadenylate could, as Allfrey[26] has suggested, be a storage form for nuclear ATP, regenerating the latter upon demand. We had considered and abandoned this hypothesis because of the low concentration of polyadenylate relative to acid-soluble adenylates. (*b*) Polyadenylate and other homopolymers could be intermediates capable of being utilized in the synthesis of more complex polynucleotides. There is no evidence for such a mechanism which lies far outside current ideas concerning the mechanism of RNA synthesis. (*c*) Perhaps the most fruitful area for investigation at present concerns a possible role for polyadenylate in the control of protein synthesis, either directly by interaction with chromosomal or ribosomal sites or indirectly through the synthesis of polylysine which in turn may exert a control function[27,28].

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RNA Synthesis and Uptake by Liver Cells in Suspension

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While it is generally agreed that organization of cells in tissue-specific patterns confers upon the cells certain characteristics which may not be obtained when the cells are not thus organized, there has been, so far, no systematic approach towards understanding the nature of those biochemical properties of cells in organized tissues which are a function of intercellular organization. One approach towards gaining useful information in regard to the biochemical basis of intercellular organization would be to compare the various biochemical properties of tissue slices with those of single-cell suspensions derived from the same tissue, and to see to what extent the biochemical changes obtained on dispersion of the tissue to a single cell suspension can be reversed on reaggregation of the cell suspension to tissue-like structures[1,2].

For the above studies it is necessary that the single cell suspensions be obtained by a method which causes only those changes in the biochemical properties of the cells which are intrinsic to disruption of the intercellular organization and which are not caused by other physical or chemical damage to the cells due to inadequacies of the method used for the dispersion of the tissue. We have recently described a method for the preparation of hepatic cell suspensions from rat liver which seems to satisfy the above criterion[3,4].

This method uses a tissue disperser consisting of a loosely-fitting rubber pestle in a hard glass tube, and (i) gives a 90% recovery of the hepatic cells of the tissue in the final cell suspension; (ii) does not use any enzymatic or drastic physical treatment; (iii) gives cell suspensions in which most of the cells exist as single cells and which are virtually free of clumps; and (iv) takes only 15 min. for the preparation of the cell suspension. Since it appeared that hepatic cell suspensions obtained by this technique could form a good system for gaining information on the biochemical basis of intercellular organization, we undertook a systematic investigation of the similarities and the differences in the biochemical properties of the liver tissue slices and the cell suspensions. In this paper, some studies on the synthesis and uptake of ribonucleic acid by hepatic cells in suspension are described. These studies clearly show that cell suspensions differ markedly from the tissue slices in the utilization of the various precursors of RNA, in the effect of various inhibitors on RNA synthesis, and in the ability to effect the transport of macromolecular RNA.

EXPERIMENTAL PROCEDURE

The rat hepatic cell suspensions were prepared by the method of Jacob and Bhargava[3]. The incubation media were prepared according to Dawson *et al*[5]. The following abbreviations have been used for these media: KRP buffer=Krebs-Ringer phosphate buffer; KRB buffer=Krebs-Ringer bicarbonate buffer; and TYR solution=Tyrode solution.

In experiments on the incorporation of various precursors [(^{32}P) phosphate, (8-^{14}C) adenine, (2-^{14}C) uracil and (6-^{14}C) orotic acid] into RNA, and the effect of chloramphenicol, ribonuclease and actinomycin D on this incorporation, the tissue slices (10-30 mg. dry wt) or the hepatic cells in suspension (6-25 million cells; 9-38 mg. dry wt) were incubated with the precursor or/and inhibitor in 2-4 ml. of the medium at 37°C. with shaking. The total nucleic acid fraction was isolated by either the sodium chloride extraction of the TCA-precipitate with (Method I) or without (Method II) prior extraction of lipids, or by the method used by Iverson and Giese[6]. RNA was obtained in the hydrolysed form from the total nucleic acid fraction by treatment with alkali. The separation of nucleotides in the alkaline hydrolysate was done by electrophoresis[7]. In experiments in which it was desired to determine the specific activity of the individual bases, the total nucleic acid fraction was hydrolysed with 72% perchloric acid and the bases separated by chromatography[8]. An optical density of 1·0 in the neutralized RNA fraction after alkaline hydrolysis was taken to correspond to 33 μg. of RNA/ml. In solutions containing unhydrolysed mixed nucleic acids (RNA+DNA), an optical density of 1·0 was taken to correspond to 40 μg. of total nucleic acid/ml.; these values are based on an increase of 22% in the absorbancy of rat liver RNA on alkaline hydrolysis[9].

In experiments on the uptake of macromolecular RNA, RNA was isolated by the phenol-extraction procedure from the freshly excised liver of a rat which had been injected with labelled phosphate 24 hr earlier, and the hepatic cells in suspension derived from another animal incubated with this (labelled) RNA. At the end of the incubation period, the extracellular labelled RNA was removed by extensive washing, and the specific activity of RNA obtained in the hydrolysed form from the total nucleic acids extracted from the cell suspension as described above, was determined.

Other relevant experimental details are given at the appropriate place in the tables, figures and the text that follows.

Synthesis of RNA from Various Precursors by Hepatic Cell Suspensions

The incorporation of phosphate into the RNA of the cell suspensions was of the same order of magnitude and followed the same pattern as was obtained in the case of the perfused or the unperfused slices (Fig. 1). Alkaline hydrolysis of the total nucleic acid fraction obtained by sodium chloride extraction of lipid-free TCA precipitate, and purification of the ribonucleotides by electrophoresis, showed that highly labelled ^{32}P-containing contaminants[10,11] were present, if at all, in the RNA fraction, in a quantity which was insignificant and lower than that obtained in the case of the slices. The variation from experiment to experiment in the extent of the uptake of the labelled precursor into RNA was much more in the cell suspensions than in the tissue slices.

Adenine was not incorporated into the RNA of cell suspensions, unlike into the RNA of the slices, when the incubation medium was TYR solution, in which the phosphate concentration is low; there was substantial RNA synthesis from labelled adenine in the cell suspensions incubated in KRP buffer in which the phosphate concentration is about 50 times as high as in TYR solution (Table 1). The cell suspensions differed from the tissue slices also in the following significant respects: (i) the cell suspensions, unlike the slices, exhibited a marked lag (Fig. 1) in the incorporation of adenine into RNA; and (ii) the incorporation in the cell suspensions was stimulated significantly by the addition of glucose to the incubation medium, while there was no such stimulation in the slices (Table 1). The latter observation indicates that cell suspensions may be unable to use their glycogen reserves and that in the cell suspensions, either the transport of labelled adenine or/and the conversion of intracellular adenine to adenosine phosphates may proceed at a much slower rate than in the tissue slices. As a result of the initial lag, the incorporation in the cell suspensions at shorter time intervals (e.g. 2 hr) was much less than in the tissue slices, although the incorporation at 4 hr was usually more in the cell suspensions than in the tissue slices (Table 1; Fig. 2). With labelled adenine as the precursor, in both the tissue preparations, the guanine of RNA was labelled to an extent of approximately 10% of the label obtained in RNA-adenine.

The two tissue preparations differed significantly in respect of incorporation of orotic acid into RNA; while the tissue slices incorporated this precursor into their RNA in significant amounts in every experiment, no detectable incorporation was obtained in the cell suspension in any of a series of 10 experiments, excepting one, in which too the incorporation in the cell suspensions was only 5% of that obtained in the slices (Table 2). In this experiment, in both the tissue preparations, the cytosine of RNA was labelled to an extent of about 50% of the label obtained in RNA-uracil.

The loss of the ability to incorporate orotic acid on dispersion of the

TABLE 1—INCORPORATION OF (8-^{14}C) ADENINE INTO THE RNA OF RAT LIVER SLICES AND CELL SUSPENSIONS

[(^{14}C) Adenine concentration was 0·46 μC (0·13 μmoles)/ml.]

Exp. No.	Incubation medium	Specific activity of RNA (c.p.m./mg.)					
		Cell suspensions			Perfused liver slices		
		120 min.	180 min.	240 min.	120 min.	180 min.	240 min.
74*	TYR	0	0	0	338	402	435
76	KRP	194	576	1268	402	634	820
	KRP†	145	388	404	410	641	855
73	KRP	95	..	1471	500	..	707

*Specific activity values are for mixed nucleic acid (RNA+DNA) preparation.
†Without glucose.

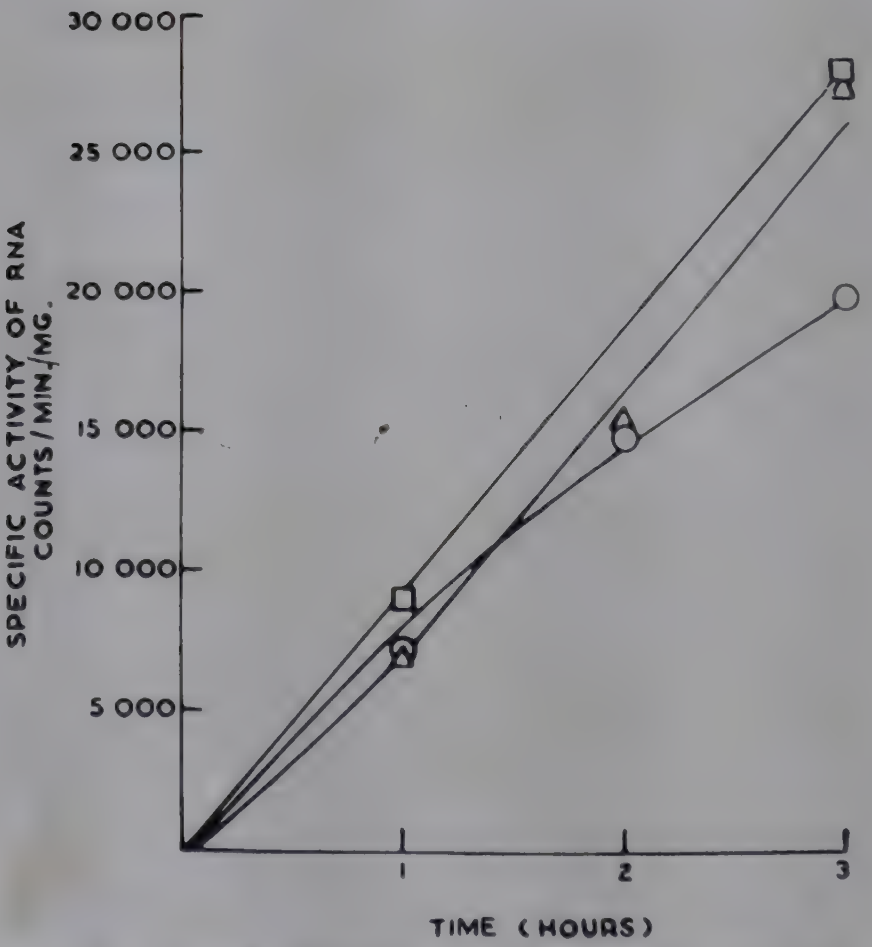

FIG. 1—INCORPORATION OF (^{32}P) PHOSPHATE INTO RNA OF RAT LIVER CELL SUSPENSIONS (O), PERFUSED LIVER SLICES (△) AND UNPERFUSED LIVER SLICES (□): (^{32}P) Phosphate concentration including the phosphate of the buffer) was 33·3 μC (69·8 μg.)/ml.

TABLE 2—Incorporation of (6-^{14}C) Orotic Acid into the RNA of Rat Liver Slices and Cell Suspensions

[(^{14}C) Orotic acid concentration was 0·67 μC (0·066 μmoles)/ml.]

Exp. No.	Incubation medium	Specific activity of RNA (c.p.m./mg.)					
		Cell suspensions			Perfused liver slices		
		120 min.	180 min.	240 min.	120 min.	180 min.	240 min.
49	TYR	0*	0	..	61*	185	..
	TYR	..	..	..	290†	613†	..
82	TYR	0	..	0	161	..	236
	KRP	0	..	0	695	..	1001
	KRP‡	0	..	0	470	..	1341
79§	KRP	..	71	127	1935	2261	2481

*90 min.
†Unperfused slices.
‡Without glucose.
§Specific activity values are for mixed nucleic acid (RNA+DNA) preparation.

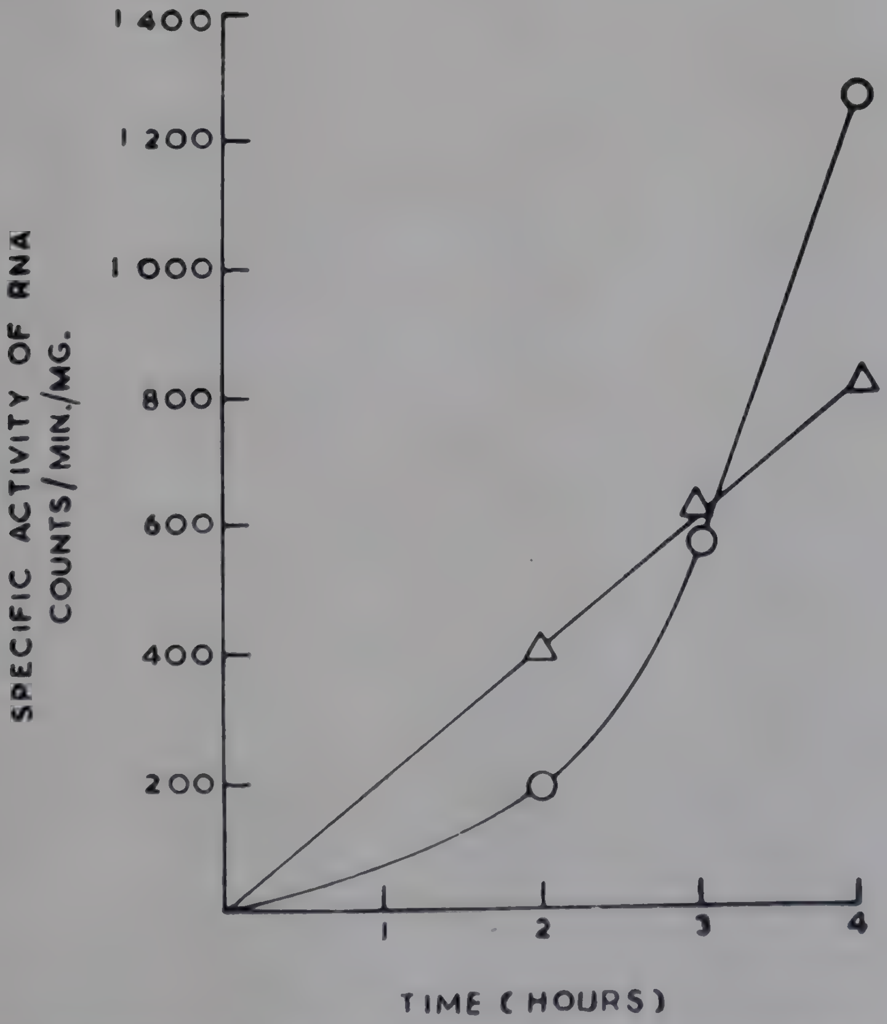

Fig. 2—Incorporation of (^{14}C) adenine into the RNA of rat liver cell suspensions (O) and perfused liver slices (△): (^{14}C)Adenine concentration was 0·46 μC (0·13 μmoles)/ml.

liver tissue led us to study the incorporation of uracil into RNA of the cell suspensions, which in certain mammalian systems, like malignant tissues, acts as a good (even preferred) precursor of RNA-pyrimidines[12]. It was found that as in tumour tissues, the hepatic cells in suspension incorporated uracil to a much higher extent than the liver tissue slices (Table 3). It has been recently shown by Canellakis[13] that the enhanced ability of tumour tissues to utilize exogenous (2-^{14}C) uracil for RNA-pyrimidine synthesis is due to the absence in the tumours of the enzyme system which can convert the 2-carbon of uracil to CO_2; this enzyme system is present in normal liver and causes degradation of much of the labelled precursor to labelled carbon dioxide, unless the precursor is used at a very high concentration. We have

TABLE 3—Incorporation of (2-^{14}C) Uracil into the RNA of Rat Liver Slices and Cell Suspensions

[Incubations were carried out in KRP buffer. (^{14}C) Uracil concentration was 0·46 μC (0·045 μmoles)/ml.]

Exp. No.	Specific activity of RNA (c.p.m./mg.)			
	Cell suspensions		Perfused liver slices	
	120 min.	240 min.	120 min.	240 min.
87	54	669	31	213
105	32	366	38	90
108	44	523	..	600
111	..	850	..	123
139*	..	5700	..	1840

*Ca^{2+}-free KRP.

TABLE 4—Formation of $^{14}CO_2$ from (2-^{14}C) Uracil

[Incubations were carried out in Ca-free KRP buffer in a Warburg flask for 240 min. (^{14}C) uracil concentration was 0·46 μC (0·045 μmoles)/ml. The CO_2 evolved was trapped in alkali and counted as $BaCO_3$]

Tissue preparation	Weight of TCA-insoluble ppt. derived from the whole of the tissue preparation incubated with precursor (mg.) "A"	Total radioactivity in CO_2 evolved during incubation (c.p.m.) "B"	Radioactivity in CO_2 evolved (c.p.m.)/mg. (TCA-insol. ppt.) "B"/"A"
Unperfused slices	36·7	295600	8000
Perfused slices	35·7	266500	7465
Cell suspensions	100·5	5830	58

also found that hepatic cell suspensions, unlike the normal liver tissue but like malignant tissues, are unable to degrade (2-^{14}C) uracil to labelled carbon dioxide (Table 4). It would, therefore, appear that dispersion of the liver tissue to single cell suspensions causes either a leakage or inactivation of one or more of the enzymes involved in the above degradation.

The above observations on the incorporation of various precursors into the RNA of the two tissue preparations show that while hepatic cells in suspension are capable of synthesizing RNA, there are significant difference between the two tissue preparations in the manner and extent of the incorporation of the various precursors.

Effect of Inhibitors on RNA Synthesis by Hepatic Cells in Suspension

Effect of chloramphenicol. Chloramphenicol, which does not inhibit the incorporation of adenine and uracil into RNA in liver slices, almost completely inhibited the incorporation of these two bases into the RNA of liver cell suspensions at a concentration of 50 μg./ml., both in experiments in which the radioactivity of the total RNA of the cells was estimated, as well as those in which the cells, after incubation with the precursor and removal of the extracellular precursor by adequate washing of the cells, were fractionated into the usual subcellular fractions and the radioactivity in the RNA of each of these fractions was determined (Tables 5-8). Chloramphenicol, however, inhibited the incorporation of both adenine and uracil into the RNA of the homogenate derived from the perfused whole liver tissue, as in the case of the whole cell suspensions or their

TABLE 5—The Effect of Chloramphenicol on the Incorporation of (8-^{14}C) Adenine into the RNA of Rat Liver Slices and Cell Suspensions

[Incubations were carried out in Ca-free KRP buffer for 240 min. The concentrations of chloramphenicol (CLP) and adenine were 50 μg./ml. and 0·45 μC (0·49 μmoles)/ml. respectively]

Exp. No.	Specific activity of RNA (c.p.m./mg.)			
	Cell suspensions		Perfused liver slices	
	−CLP	+CLP	−CLP	+CLP
122	622	33	57	67
123	1790	23	..	..
125	..	..	223	184
137	6300	32	..	..
141	..	..	770	920

TABLE 6—THE EFFECT OF CHLORAMPHENICOL ON THE INCORPORATION OF (^{14}C)ADENINE INTO THE NUCLEIC ACIDS AND THE TCA-SOLUBLE FRACTION OF THE SUBCELLULAR FRACTIONS DERIVED FROM RAT LIVER SLICES AND CELL SUSPENSIONS

(Incubations were carried out in 8 ml. of Ca-free KRP buffer for 240 min. The concentrations of chloramphenicol and adenine were as in Table 5. The tissue preparation was washed adequately after incubation to remove adhering labelled precursor. The subcellular fractions, and the total nucleic acid and acid-soluble fractions were isolated in the usual way)

Tissue preparation	Cell fraction	Change in specific activity (c.p.m./mg.) of nucleic acids with chloramphenicol	Change in total activity (c.p.m.) in TCA-soluble fraction with chloramphenicol
Perfused liver slices	Nuclei	410→312 (−24%)	2045→1858 (− 9%)
	Mitochondria	171→148 (−13%)	3106→3346 (+ 8%)
	Microsomes	50→ 44 (−12%)	349→ 309 (−11%)
	Supernatant	344→294 (−14%)	759→ 744 (− 2%)
Cell suspensions	Nuclei	1450→ 60 (−96%)	1268→ 808 (−36%)
	Mitochondria	1220→ 32 (−97%)	1316→ 876 (−33%)
	Microsomes	0→ 0	142→ 87 (−39%)
	Supernatant	517→ 70 (−86%)	534→ 864 (+62%)

TABLE 7—THE EFFECT OF CHLORAMPHENICOL ON THE INCORPORATION OF (2-^{14}C) URACIL INTO THE RNA OF RAT LIVER CELL SUSPENSIONS

[Incubations were carried out in Ca-free KRP buffer for 240 min. The concentrations of chloramphenicol (CLP) and (^{14}C) uracil were 50 μg./ml. and 0·46 μC (0·045 μmoles/ml.)]

Exp. No.	Specific activity of RNA (c.p.m./mg.)			
	Cell suspensions		Perfused liver slices	
	−CLP	+CLP	−CLP	+CLP
131	4860	0	0	13
139	5700	25	..	..
140	2300	20	..	..
141	..	..	320	240

homogenate (Table 9). This shows that the lack of inhibition by chloramphenicol of RNA synthesis from the free bases in the tissue slices, in contrast to the complete inhibition in the cell suspensions, is probably due to permeability barriers existing in tissue slices which are lost on dispersion of the tissue to single cell suspensions. In microorganisms which are permeable to chloramphenicol, this antibiotic has been shown to inhibit the incorporation of labelled adenine and uracil into RNA[14,15]. This appears to be the first report in which chloramphenicol, at low concentrations, has been shown to have an inhibiting effect on some steps in RNA synthesis in a whole-cell mammalian system.

The inhibitory effect of chloramphenicol on the incorporation of

TABLE 8—THE EFFECT OF CHLORAMPHENICOL ON THE INCORPORATION OF (^{14}C) URACIL INTO THE NUCLEIC ACIDS AND TCA-SOLUBLE FRACTIONS OF THE SUBCELLULAR FRACTIONS DERIVED FROM RAT LIVER SLICES AND CELL SUSPENSIONS

[Chloramphenicol and (^{14}C) uracil concentrations were as in Table 7. Other details were as in Table 6]

Tissue preparation	Cell fraction	Change in specific activity (c.p.m./mg.) of nucleic acids with chloramphenicol	Change in total activity (c.p.m.) in TCA-soluble fraction with chloramphenicol
Perfused liver slices	Nuclei	116→114 (− 1%)	579→ 540 (− 7%)
	Mitochondria	54→ 55 (+ 2%)	869→ 842 (− 2%)
	Microsomes	53→ 50 (− 6%)	372→ 364 (− 2%)
	Supernatant	0→ 0	84→ 88 (+ 5%)
Cell suspensions	Nuclei	564→ 48 (−92%)	570→ 540 (− 7%)
	Mitochondria	370→ 86 (−77%)	1383→1153 (−17%)
	Microsomes	105→ 0 (−100%)	208→ 140 (−30%)
	Supernatant	443→ 70 (−84%)	543→ 978 (+80%)

TABLE 9—THE EFFECT OF CHLORAMPHENICOL ON THE INCORPORATION OF (^{14}C) ADENINE AND (^{14}C) URACIL INTO THE RNA OF RAT LIVER CELL SUSPENSIONS AND OF HOMOGENATES DERIVED FROM THE CELL SUSPENSIONS AND PERFUSED LIVER

[Incubations were carried out in Ca-free KRP buffer for 240 min. (^{14}C) adenine and (^{14}C) uracil concentrations were 0·45 μC (0·49 μmoles)/ml. and 0·45 μC (0·045 μmoles)/ml. respectively. The concentration of chloramphenicol (CLP) was 50 μg./ml.]

Precursor	Specific activity of RNA (c.p.m./mg.)				
	Cell suspensions		Homogenate from cell suspensions	Homogenate from perfused liver	
	−CLP	+CLP	+CLP	−CLP	+CLP
(^{14}C) Adenine	2172	55	117	2330	94
(^{14}C) Uracil	650	25	33	161	29

adenine and uracil into RNA in the cell suspensions could not be due to inhibition of the transport of the bases, since (i) the effect was also obtained in homogenates; and (ii) in most experiments, chloramphenicol caused no or very little reduction in the total radioactivity in the acid-soluble fraction of the cells in suspension, in the case of both the precursors (Tables 6 and 8); occasionally, there was a slight reduction, but in no case was it sufficient to account for the almost complete inhibition of the incorporation of the isotope into RNA. Since free bases are not the normal precursors of RNA purines and pyrimidines, at least in mammalian tissues, it appeared possible that chloramphenicol was inhibiting some steps in the conversion of the base to the nearest precursor (the nucleoside monophosphate) formed as an intermediate in the normal biosynthetic pathway leading to the incorporation of the free base into RNA. If this were true, chloramphenicol will not be expected to inhibit net RNA synthesis, which could proceed through the normal pathways (which do not involve the free base at any stage) by which purine and pyrimidine nucleotides are formed in mammalian systems. The above mentioned possibility is strongly supported by a large number of earlier investigations in which chloramphenicol has been shown to have no effect on RNA synthesis in various systems, when the synthesis is measured either by determining the net increase in the amount of RNA or by estimating the incorporation of labelled phosphate, uridine, guanine or glycine into RNA[14,16–28]. In this investigation as well, chloramphenicol did not inhibit (in fact it stimulated) the incorporation of labelled phosphate into RNA of the cell suspensions and the whole tissue homogenates as in the case of the tissue slices (Table 10).

The results of this investigation coupled with those of the earlier ones in which the effect of chloramphenicol on net RNA synthesis or on the incorporation of various precursors into RNA has been studied, allow the following conclusion to be drawn: in those systems, bacterial or mammalian, in which no permeability barriers towards chloramphenicol exist, this antibiotic, while having no effect on the net synthesis of RNA by the normal biosynthetic pathways (which do not involve a free base at any stage) in the cell, completely inhibits the conversion of free adenine or uracil to RNA purines or pyrimidines, by blocking the direct conversion of the base to the corresponding nucleoside monophosphate.

Effect of ribonuclease. When the hepatic cell suspensions were incubated with ribonuclease (100 μg./ml.), 60-80 per cent of their RNA was found to be degraded in 3-4 hr, while no such degradation was observed in the presence (or the absence) of ribonuclease in the tissue slices (Table 11). This shows that dispersion of liver tissue makes the hepatic cells permeable to ribonuclease.

The specific activity of the residual RNA in the hepatic cells in suspension incubated with various precursors of RNA in the presence of ribonuclease, was always significantly higher than that of the total cellular RNA obtained from cells incubated in the absence of externally added ribonuclease (Table 12). This strongly indicates that ribonuclease preferentially degrades a RNA(s) which is relatively metabolically inert, and that the metabolically more active RNA of the cells in suspension is relatively resistant to ribonuclease.

Effect of actinomycin D. Actinomycin D, at concentrations of 0·03-1·10 μg./ml., inhibited the incorporation of labelled phosphate or adenine into RNA in perfused liver slices to an extent of 30-70% showing that the hepatic cells in the organized tissue structure are permeable to actinomycin D at high extracellular concentrations of the antibiotic (Table 13). However, in the cell suspensions actinomycin D had no effect whatsoever on the incorporation of the above precursors into RNA. This

TABLE 10—THE EFFECT OF CHLORAMPHENICOL ON THE INCORPORATION OF (^{32}P) PHOSPHATE INTO THE RNA OF RAT LIVER SLICES, HOMOGENATES AND CELL SUSPENSIONS

[Incubations were carried out in KRB buffer. (^{32}P) phosphate concentration was 33·3 μC (69·8 μg. P)/ml. The concentration of chloramphenicol (CLP) was 50 μg./ml.]

Incubation Period min.	Specific activity of RNA (c.p.m./mg.)					
	Cell suspensions		Perfused liver slices		Homogenate from perfused liver	
	−CLP	+CLP	−CLP	+CLP	−CLP	+CLP
60	1134	2830	2601	2413	6604	10220
120	2176	5190	5510	8567	6661	16500
180	2750	6970	6898	17340	7725	30810

TABLE 11—RECOVERY OF RNA AFTER TREATMENT WITH RIBONUCLEASE

[Incubations were carried out in Ca-free KRP buffer. (^{14}C) uracil concentration was 0·46 μC (0·045 μmoles)/ml. The concentration of ribonuclease was 100 μg./ml.]

Exp. No.	Percent reduction in the amount of RNA			
	Cell suspensions		Perfused liver slices	
	120 min.	240 min.	120 min.	240 min.
106	51	80	8	30
109	64	69	13	..
111	49	60	1	10
112*	..	80	..	7
116	..	80	..	..

* With Ca^{2+}

may be due to one of the following two reasons: (i) Dispersion of the hepatic tissue may cause a reduction in the permeability of the hepatic cells to actinomycin D. (ii) In the cell suspensions, all the RNA that is synthesized may be made by a DNA-independent mechanism. It is possible that such a mechanism may be responsible for the synthesis of a small amount of RNA even in the organized hepatic tissue; during the dispersion of the tissue one of the key enzymes, e.g. RNA polymerase, involved in the DNA-dependent RNA synthesis, may leak out of the cell, thereby making the entire RNA

TABLE 12—THE EFFECT OF RIBONUCLEASE ON THE INCORPORATION OF (^{14}C) URACIL AND (^{32}P) PHOSPHATE INTO THE RNA OF RAT LIVER SLICES AND CELL SUSPENSIONS

[Incubations were carried out in Ca-free buffer. The concentrations of (^{14}C) uracil and (^{32}P) phosphate were 0·46 μC (0·045 μmoles)/ml. and 33·3 μC (69·8 μg.P)/ml. respectively. The concentration of ribonuclease was 100 μg./ml.]

Precursor	Time min.	Change in specific activity (c.p.m./mg.) of RNA with ribonuclease	
		Cell suspensions	Perfused liver slices
(^{14}C) Uracil	120	43→ 93 (+116%)	38→ 36 (−6%)
	240	93→ 302 (+212%)	85→ 79 (−7%)
(^{32}P) Phosphate	180	7930→14465 (+ 82%)	11085→11245 (×1%)

TABLE 13—THE EFFECT OF ACTINOMYCIN D ON THE INCORPORATION OF (8-^{14}C) ADENINE AND (^{32}P) PHOSPHATE INTO THE RNA OF RAT LIVER SLICES AND CELL SUSPENSIONS

[Incubations were carried out in KRP buffer in case of phosphate and Ca-free KRP buffer in case of adenine. (^{14}C) adenine and (^{32}P) phosphate concentrations were 0·45 μC (0·49 μmoles) and 0·25 μC (61·8 μg.P)/ml. respectively]

Conc. of actinomycin D μg./ml.	Precursor	Time min.	Percentage reduction in specific activity of RNA	
			Cell suspensions	Perfused liver slices
0·03	(^{14}C) adenine	240	0	37
0·03	(^{32}P) phosphate	180	0	40
0·19	(^{14}C) adenine	240	0	33
0·19	(^{14}C) adenine	240	0	46
0·56	(^{14}C) adenine	240	0	70
1·10	(^{14}C) adenine	240	0	55

synthesis in the cell suspension DNA-independent and, therefore, actinomycin D insensitive.

Further work is in progress to check which one of these alternatives holds true.

The above studies on the effect of inhibitors clearly indicate that hepatic cells in suspension could form an excellent material for gaining fundamental information in respect of the metabolism of RNA and the mechanism of its biosynthesis.

UPTAKE OF MACROMOLECULAR RNA BY HEPATIC CELLS IN SUSPENSION

While the uptake of DNA in bacterial cells, and in recent years in mammalian cells, has been studied extensively, there have been only a few investigations on the *in vitro* uptake of RNA by mammalian cells; in some of these studies, induction of protein synthesis by the exogenous RNA[29–35] has been demonstrated. Almost all these studies have been confined to either cells grown in tissue culture, or to cell systems which occur naturally as cell suspensions and, in this respect, resemble those systems in which transformation with DNA has been studied. Further, the fate of the transported RNA was not investigated. On account of the permeability changes, reported in this investigation, which were found to occur on dispersion of liver tissue to single cell suspensions, it seemed possible that the hepatic cells in suspension may be permeable to RNA while the tissue slices are not. In view of these considerations, it was considered to be of interest to study the uptake of homologous, labelled, macromolecular RNA by hepatic cells in suspension.

Very little uptake of radioactivity was obtained in the tissue slices on incubation with ^{32}P-labelled RNA from rat liver, on the other hand, the uptake was considerable in the case of the hepatic cell suspensions (Fig. 3). The uptake in the cell suspensions progressively increased up to a period of 30 min. after which, apparently, the RNA which was taken up was progressively degraded; in the case of the tissue slices there was a constant increase with time in the small amount of radioactivity taken up by the cells. The cells in suspensions, even at zero time point, appear to have picked up considerable RNA from the extracellular medium which could not be removed by extensive washing. Ribonuclease caused a decrease in the uptake of RNA by the liver cells in suspension; in the presence of this enzyme, the pattern of the uptake of radioactivity was similar to that obtained with the slices (Table 14).

When the cell suspensions were incubated with a mixture of nucleotides and/or oligonucleotides obtained by alkaline or enzymatic hydrolysis of ^{32}P-labelled homologous RNA, there was no uptake of radioactivity in the RNA of the cell suspensions (Table 15). These experiments show that the uptake of radioactivity by hepatic cells in suspension incubated with labelled RNA (Fig. 3) is likely to be due to the transport of macromolecular RNA (not necessarily without cleavage of any internucleotide linkage or change in conformation) from outside to inside the cell. It is possible that in the organized tissue, extracellular RNA finds it difficult to get in due to the presence of the large amount of protein in the intercellular cement[36] which may bind the RNA; this could indeed be a mechanism to guard against harmful somatic transformations in higher multicellular organisms.

The amount of extracellular RNA taken up by the hepatic cells in suspension increased as the concentration of RNA in the extracellular medium was increased up to a certain level beyond which there was no further increase in the amount of RNA taken up by the cells (Table 16). At a concentration of 118 μg./ml., about 10% of RNA present in the extracellular medium was taken up by 7×10^6 cells; the above value is much

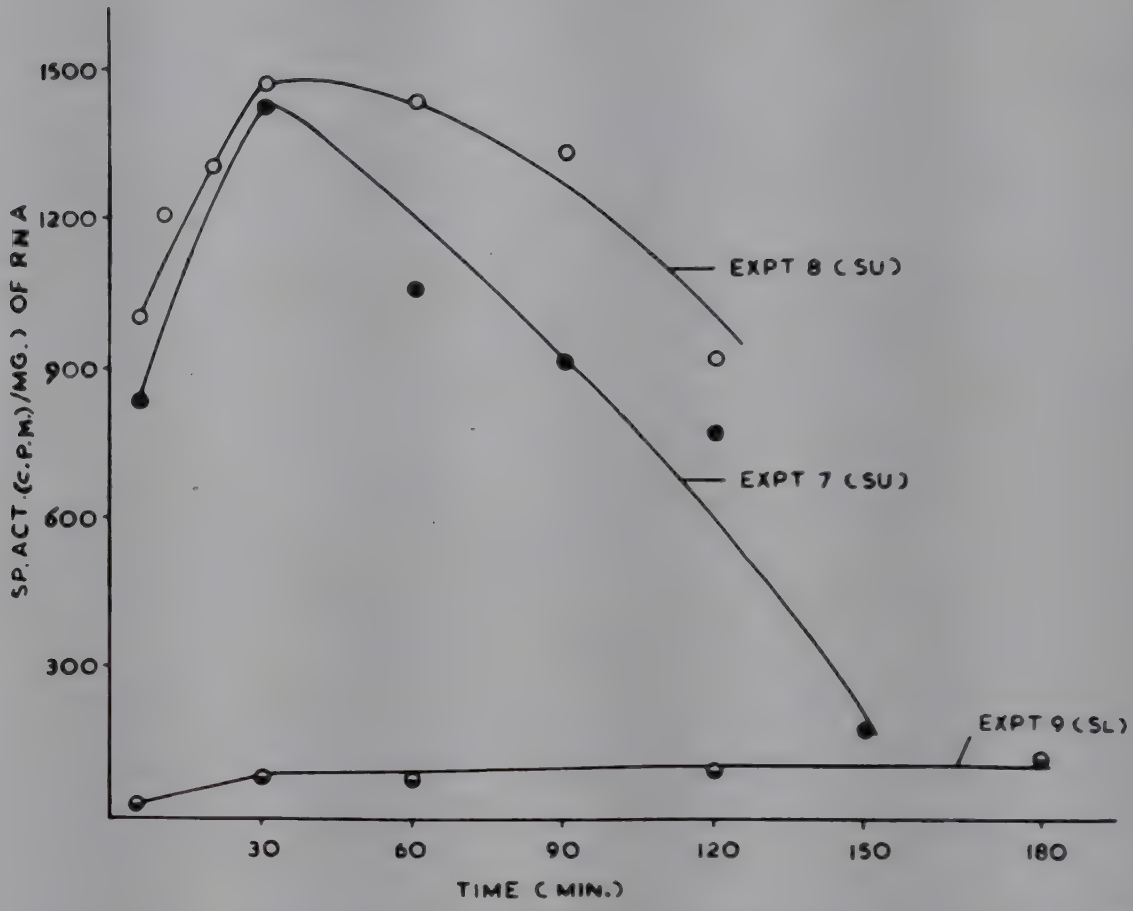

Fig. 3—Uptake of RNA by hepatic cell suspensions (○, ●), and liver slices (◒): The concentration of RNA was 280 μg./ml. in Exp. 7, 219 ug./ml. in Exp. 8, and 197 μg./ml. in Exp. 9. The specific activity of RNA was 40200, 46500, and 27680 c.p.m./mg. in Exp. 7, 8 and 9 respectively

higher than that reported earlier[29]. The ratio of RNA taken up to RNA present in the extracellular medium decreased as the concentration of RNA outside the cells was increased.

We have been, in this investigation, unable to confirm the stimulation of protein synthesis on incubation of mammalian cells in the presence of

TABLE 14—The Effect of Ribonuclease on RNA Uptake by Cell Suspensions

[Incubations were done in KRP buffer. The concentration and the specific activity of added RNA were 180 μg./ml. and 76520 counts/min./mg. respectively. The concentration of ribonuclease (RNase) was 16·7 μg./ml.]

Incubation Period min.	Specific activity of RNA isolated from the cells (c.p.m./mg.)	
	−RNase	+RNase
5	155	48 (49*)
60	894	302 (267*)
180	482	634 (676*)

*With Ca^{2+}-free KRP buffer as incubation medium.

TABLE 15—Uptake of Macromolecular RNA and Degraded RNA by Cell Suspensions

[Incubations were done in KRP buffer. The radioactivity in the incubation medium due to added RNA or its hydrolysed forms was the same]

	Specific activity of cellular RNA (c.p.m./mg.)		
	5 min.	60 min.	180 min.
RNA	1365	3185	2278
Ribonuclease hydrolysate of RNA	0	0	0
Alkali hydrolysate of RNA	0	17	0

TABLE 16—Uptake of RNA by Cell Suspensions: Effect of Concentration of Added RNA

[Incubations were done in KRP buffer for 30 min. Specific activity of added RNA was 40860 c.p.m./mg.]

RNA added μg./3 ml.	Specific activity of RNA isolated from the cells c.p.m./mg.	RNA taken up by the cells	
		μg.	% of RNA added
118·4	416	11·84	10·04
236·8	715	22·45	9·15
592·0	1153	46·00	7·82
828·8	1334	61·50	7·40
1065·6	1573	81·01	7·60

extracellular RNA, observed by earlier workers[30,32,34]. We could not obtain any stimulation of the incorporation of labelled leucine into the proteins of the liver cells in suspension, by externally added homologous RNA. When instead of leucine, a mixture of labelled amino acids (Chlorella protein hydrolysate) was used, a slight (50%) stimulation of the incorporation of the labelled amino acids into the proteins was repeatedly obtained in the presence of RNA (700 μg./ml.) in the extracellular medium, 3 hr after the incubation was commenced (Fig. 4). The significance of this small amount of stimulation is not clear, particularly since the RNA which is taken up by the cells is largely degraded within 3 hr.

The above observations show that hepatic cell suspensions can form excellent material for studies on transformation using nucleic acids in

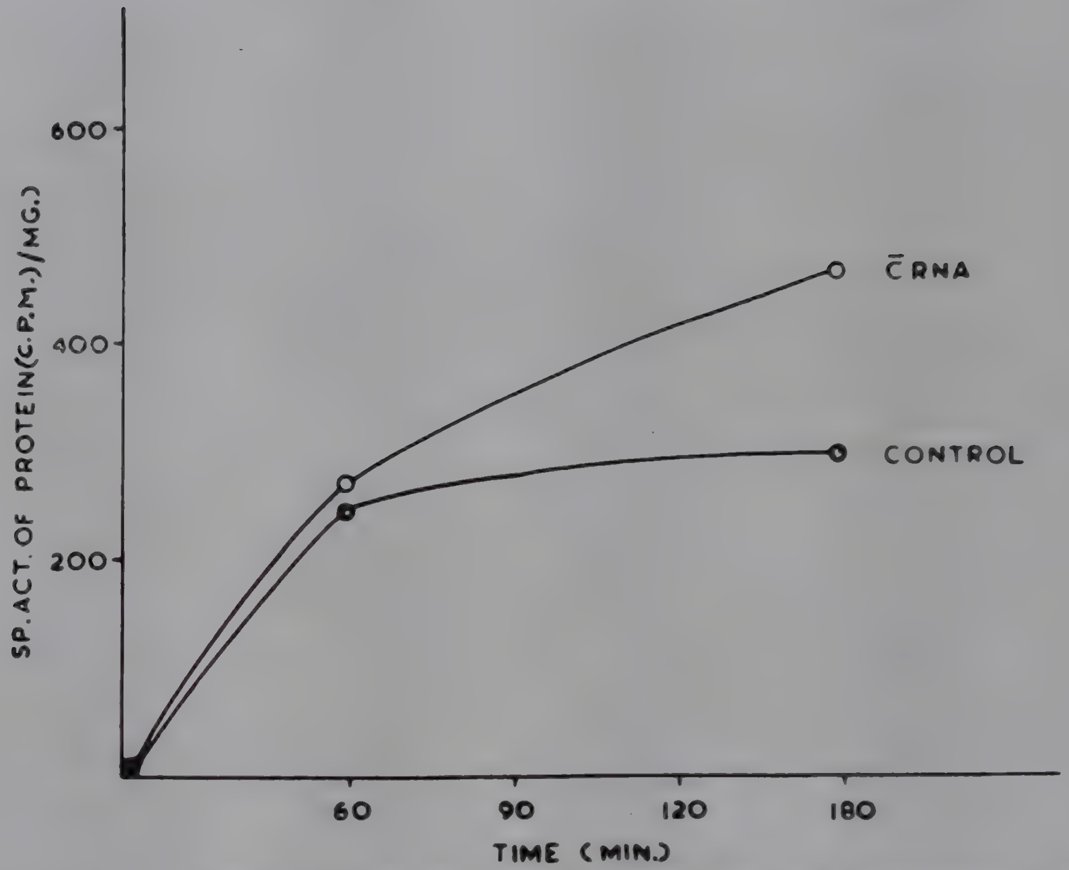

FIG. 4—INCORPORATION OF (^{14}C) AMINO ACIDS (*Chlorella* PROTEIN HYDROLYSATE) INTO PROTEINS OF CELL SUSPENSIONS WITHOUT EXOGENOUS RNA (●), AND IN PRESENCE OF EXOGENOUS RNA (O): The concentration of added RNA was 233 μg./ml. The concentration and the specific activity of (^{14}C) amino acids were 0·19 μmole and 15,000 c.p.m./ml. respectively

mammalian systems, since in cell suspensions permeability barriers which prevent the entry of RNA in the same cells when they are organized in tissues, do not exist.

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Polynucleotide Synthesis by Chloroplast Extract

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Chloroplasts are self-duplicating organelles and can synthesize RNA. When $Na_2H^{32}PO_4$ is supplied to spinach leaves, RNA in the chloroplasts is rapidly labelled and, therefore, a search has been made for RNA synthesizing systems in the chloroplast extract. The crude extract from chloroplasts has been shown to have the capacity of incorporating the four nucleoside triphosphates into polynucleotide chains[1]. Within the last few years two groups of enzymatic reactions concerning the biosynthesis of polyribonucleotides from nucleoside triphosphates have been described[2]. The first group includes RNA-polymerase and RNA-synthetase; the former is dependent on DNA and the latter on RNA[3], but both lead to the biosynthesis of RNA. The second group of enzyme reactions, which also utilizes nucleoside triphosphates, catalyzes the incorporation in independent reactions of AMP, CMP and UMP[4]. The number of newly incorporated homologous nucleotide residues varies from 2 to 100. It seems that the reactions are dependent on the presence of primer; mostly it is RNA or a homopolymer of nucleotides. DNA-dependent poly A and poly U synthesis has also been reported from different laboratories[5–7]. The separation of poly A from thymus nuclei has also been achieved[8]. GTP incorporation into poly G with nuclear extract has recently been recorded in our laboratory[9]. Our primary interest was to study the mechanism of RNA synthesis in chloroplasts but instead we have obtained evidence for several reactions which can lead to the synthesis of polyribonucleotides. In this connection we wish to describe an enzyme system which can simultaneously catalyze the incorporation of ATP and GTP into polynucleotide chains.

Polynucleotide synthesis

Chloroplasts were isolated from spinach leaves as previously described[1]. Frozen chloroplasts (10 g.) were disrupted with 20 vol. of distilled water by constant stirring for 90 min. in the cold and centrifuged at 10,000 *g* for 30 min. The pellet constitutes the grana and the supernatant stroma.

Grana were then extracted with alumina after grinding in the cold. The slurry was centrifuged at 10,000 *g* for 30 min. The supernatant was made to 0-30% saturation with $(NH_4)_2SO_4$, centrifuged and the precipitate was dispersed in 0·1*M* Tris buffer (*p*H 7·5) and dialyzed overnight in the cold. This was used as the source of enzyme without further purification.

When the crude extract of whole chloroplasts was incubated with four ribonucleoside triphosphates individually or together, it was found that all the four triphosphates were incorporated. But in the presence of other nucleoside triphosphates, ATP incorporation was not increased and was instead decreased. The data are given in Table 1. This inhibition of ATP incorporation was further followed up; the extract from grana after partial purification showed maximum incorporation of ATP and GTP but CTP and UTP incorporation was reduced to nil. Another consistent observation was the inhibition of ATP by GTP and vice versa. ATP or GTP incorporation was not inhibited by other triphosphates. The data are given in Table 2. The next problem investigated was on the nature of the substrate, and this was done by studying the effect of PP_i, AMP and ADP on ATP incorporation and of GMP and GDP on GTP incorporation. From the data presented in Table 3, it is apparent that (i) pyrophosphate is inhibitory, (ii) ATP might be degraded to ADP by ATPase and (iii) ADP might be incorporated through polynucleotide phosphorylase. However, it was observed that inorganic phosphate was not inhibitory even at a high concentration. When ATP was converted to ADP by treatment with hexokinase (10 μg./ml.), the incorporation was reduced to 60%; the incorporation should have increased if it were due to polynucleotide phosphorylase. The other alternative is to assume that adenylate kinase activity is associated with the preparation. As a matter of fact when the preparation was assayed for kinase activity it was found to be positive. Furthermore, GDP had no inhibitory effect on ATP incorporation. The effect of GDP and GMP on GTP

TABLE 1—Incorporation of Ribonucleoside Triphosphates by Chloroplast Extract

Substrate*	mμmoles/mg. P
ATP-8-^{14}C	0·40
GTP-8-^{14}C	0·37
CTP-2-^{14}C	0·35
UTP-2-^{14}C	0·32
ATP-8-^{14}C+GTP+UTP+CTP	0·20

*Incubation mixture contained the following: Tris (*p*H 8·0), 100 μmoles; $MgCl_2$, 5 μmoles; NTP-^{14}C, 0·5 μmole each of specific activity 8×10^5 c.p.m./μmole; 4-5 mg. protein in a total vol. of 1 ml. Incubation for 15 min. at 37°C.

TABLE 2—Incorporation of Ribonucleoside Triphosphates by Grana Extract

Substrate	mμmoles/mg. P
ATP*	0·26
GTP*	0·24
CTP*	0·00
UTP*	0·00
ATP*+GTP	0·13
GTP*+ATP	0·14
ATP*+GTP+UTP	0·11
ATP+GTP+UTP+CTP	0·11
ATP*+UTP	0·24

TABLE 3—Effect of ADP, AMP and PP_i on ATP Incorporation

Substrate	mμmoles/mg. P
ATP*	0·30
ATP*+ADP	0·14
ATP*+AMP	0·12
ATP*+glucose+hexokinase	0·15
ATP*+PP_i	0·07

TABLE 4—Effect of GDP and GMP on GTP Incorporation

Substrate	mμmoles/mg. P
Complete system*	0·30
Complete system+GDP	0·28
Complete system+GMP	0·30

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* Complete system contained the following: Tris (*p*H 7·2), 50 μmoles; $MgCl_2$, 3 μmoles; ATP, 2 μmoles; GTP, 1 μmole; PEP ,2 μmoles; glycine-^{14}C, 0·5 μmole of specific activity $1{\cdot}6 \times 10^6$ c.p.m./μmole; pyruvate kinase, 0·2 mg.; RNA particles, 3-4 mg.; *p*H 5 fraction, 1·2 mg.; and enzyme extract, 4 O.D. units/ml.

incorporation (Table 4) also supports the conclusion that nucleoside triphosphates were the actual substrates.

The optimal *p*H was found to be broad (around 8·5) in the case of ATP incorporation (Fig. 1). In the lower *p*H range GTP incorporation was found to be more susceptible than that of ATP which might suggest that these two reactions were independent and catalyzed by two different enzyme systems. However, other kinetics of the reactions are not in favour of this conclusion. The possibility of surface adsorption of ATP and GTP at different *p*H values was ruled out. Optimal incorporation was observed within 15 min. and the incorporation was linear up to 6 mg. of protein in the incubation mixture. For maximum incorporation of ATP or GTP manganese ions were found to be most effective, whereas magnesium ions were 50% as efficient as manganese ions. Without any divalent cations, there was partial activity which could not be further reduced by EDTA as shown in Table 5. The optimum concentration of manganese was found to be 5 μmoles/ml. for ATP and 10 μmoles/ml. for GTP incorporation.

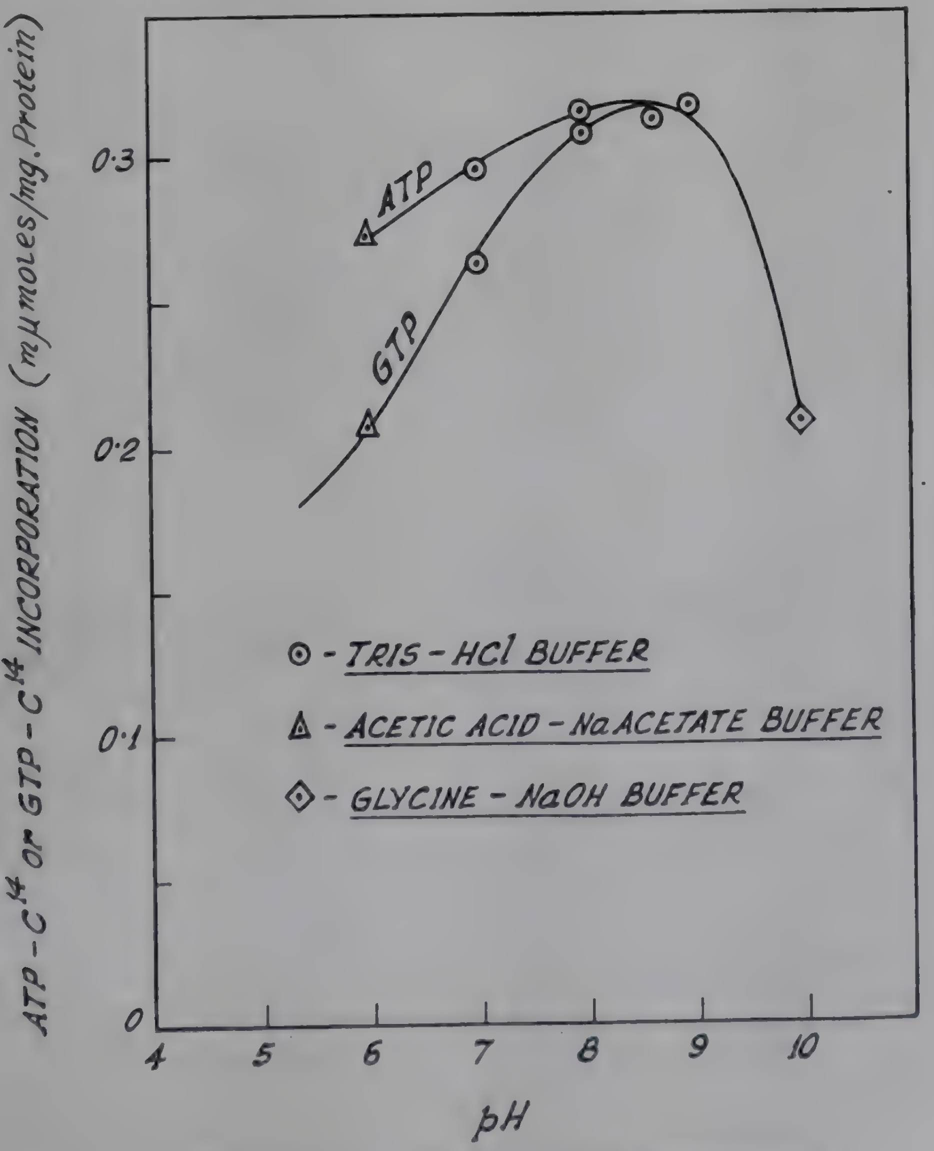

FIG. 1—*p*H OPTIMUM FOR ATP AND GTP INCORPORATION

To find out the nature of incorporation with respect to terminal and internal additions, the final product was hydrolysed and passed through Dowex-l-formate. The percentage activity in the nucleoside and nucleotide fractions is given in Table 6. At different time intervals up to 60 min., the terminal incorporation retained the same proportion suggesting that this preparation was not contaminated with terminal enzymes. The chain length of poly A which was being synthesized, if calculated from this data, would be about 30 AMP units attached together.

Effect of preincubation

It has been observed that if the enzyme was preincubated for 15 min., 40% of the activity was lost, but by the addition of poly G the activity

TABLE 5—EFFECT OF Mg^{2+} AND Mn^{2+} IONS ON ATP INCORPORATION

Metal ions	mμmoles/mg. P
—	0·18
Mg^{2+}	0·24
Mn^{2+}	0·42
EDTA	0·18

TABLE 6—PERCENTAGE OF ACTIVITY IN AMP, GMP, GUANOSINE AND ADENOSINE

	% of total activity
AMP	97
Adenosine	3
GMP	95
Guanosine	5

TABLE 7—RESTORATION OF GTP INCORPORATION BY POLY G

	mμmoles/mg. P
Preincubated enzyme (PE)	0·15
PE+RNA	0·14
PE+transfer RNA	0·17
PE+Poly C	0·13
PE+Poly G	0·26
PE+Poly A	0·18

could be restored fully. No other RNA so far tested could replace poly G. Similarly, in the case of ATP, poly A could restore the activity. The data are given in Table 7. It seems that poly G or poly A could act as acceptor RNA in respective cases. The necessity for a primer was obvious from an early lag after preincubation of the enzyme as shown in Fig. 2; this lag could be eliminated if poly G extract was added to the system.

Effect of nucleases

In order to further clarify the nature of the primer different nucleases were used; the results are given in Table 8. ATP incorporation was found to be insensitive to pancreatic RNase, DNase and T_1 RNase, but sensitive to T_2 RNase isolated from takadiastase according to the method of Takahashi[10]; this T_2 RNase can split phosphodiester bonds adjacent to adenylate. Similarly, GTP incorporation was found to be sensitive to T_1 RNase which seems to be specific for phosphodiester bonds adjacent to guanylate residues. The possibility that the slight inhibition by T_1 RNase might be due to some linkage between adenylate and guanylate residues has not yet been ruled out. It is apparent from this experiment that the primer for both the reactions is different.

Inhibition of GTP incorporation by ATP and vice versa

It has been observed that GTP incorporation was inhibited in the presence of ATP and found to be almost competitive with a purified preparation from the grana. This purification (about 4-5-fold) was achieved after

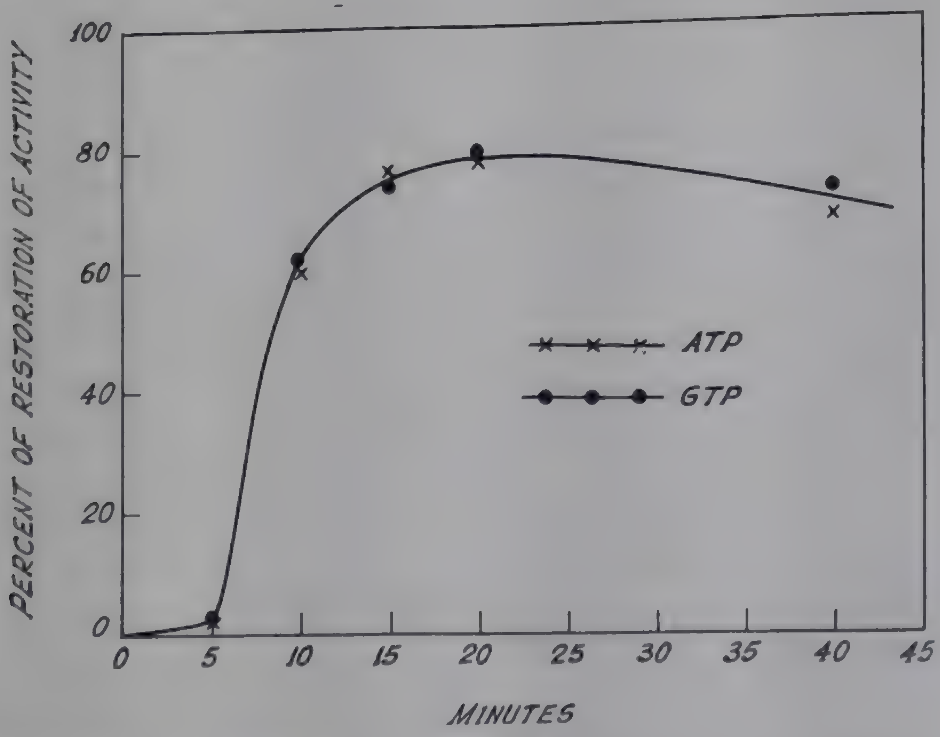

Fig. 2—Restoration of enzyme activity after pre-incubation for 15 min. by the addition of poly G extract

TABLE 8—Effect of Nucleases on ATP Incorporation

	mμmoles/mg. P
Control	0·36
Control+RNase	0·34
Control+DNase	0·34
Control+T_1 RNase	0·30
Control+T_2 RNase	0·10

TABLE 9—GTP-^{14}C Incorporation in Presence of ATP

Substrate μmoles	ATP μmoles	mμmoles/mg. P
0·235	..	0·313
0·235	0·175	0·227
0·235	0·250	0·190
0·235	0·500	0·160

washing the grana first with cold acetone till the pigments were removed; the enzyme was then extracted at 30% saturation with $(NH_4)_2SO_4$. The data are given in Table 9. ATP incorporation was similarly affected in the presence of GTP. With high amount of ATP, incorporation of GTP as has been evident from the table was not inhibited in competitive fashion. But the K_S values for GTP and ATP were found to be the same (approximately $3{\cdot}7 \times 10^{-5}$ M). When poly G or poly A was added, the affinity of the enzyme towards the substrate appeared to increase (Fig. 3); K_S value in this case was approximately $1{\cdot}1 \times 10^{-5}$ M. From the K_S values it seems that GTP and ATP incorporation is catalyzed by the same enzyme system.

Nearest neighbour frequency

The problem now was to clarify the nature of the product formed when both ATP and GTP were present in the system. For this purpose, the relative nearest neighbour frequencies were determined after incubating with AM^{32}PPP. As indicated in Table 10, about 90% of the AMP units were incorporated next to another AMP unit; this favours the synthesis of polyadenylate chains. When GTP was present there was very little change in the ApA sequence and at least 80% of the AMP units were attached to synthesize poly A. GpA sequence showed a tendency towards higher values; this does not prove that any copolymer of poly-AG is formed, but instead substantiates the conclusion that the synthesis of separate chains of poly A or poly G is favoured.

Influence of the product on amino acid incorporation

That the product is of poly A or poly G type has been proved by its characteristic influence on amino acid incorporation by RNP particles and the *p*H 5 fraction isolated from chloroplasts and also from other sources. RNP particles were isolated from stroma fraction of chloroplasts after centrifugation at 105000 *g* for 2·5 hr. Finally, the *p*H 5 fraction was isolated from the supernatant. From Table 11 it will be seen that glycine incorporation was dependent on the addition of the product containing poly G synthesized enzymatically. With double the concentration approximately two-fold increase in glycine incorporation was achieved. The incorporation of other amino acids so far tried was least affected by the addition of this poly G fraction. Similarly, lysine incorporation was increased by the product containing poly A after incubation with ATP[11]. Forty-one out of sixty-four triplets in RNA have already been decoded on the basis of data on the stimulation of the incorporation of amino acids by poly U, poly A, poly C and other copolymers. A doublet may be shared with the same relative

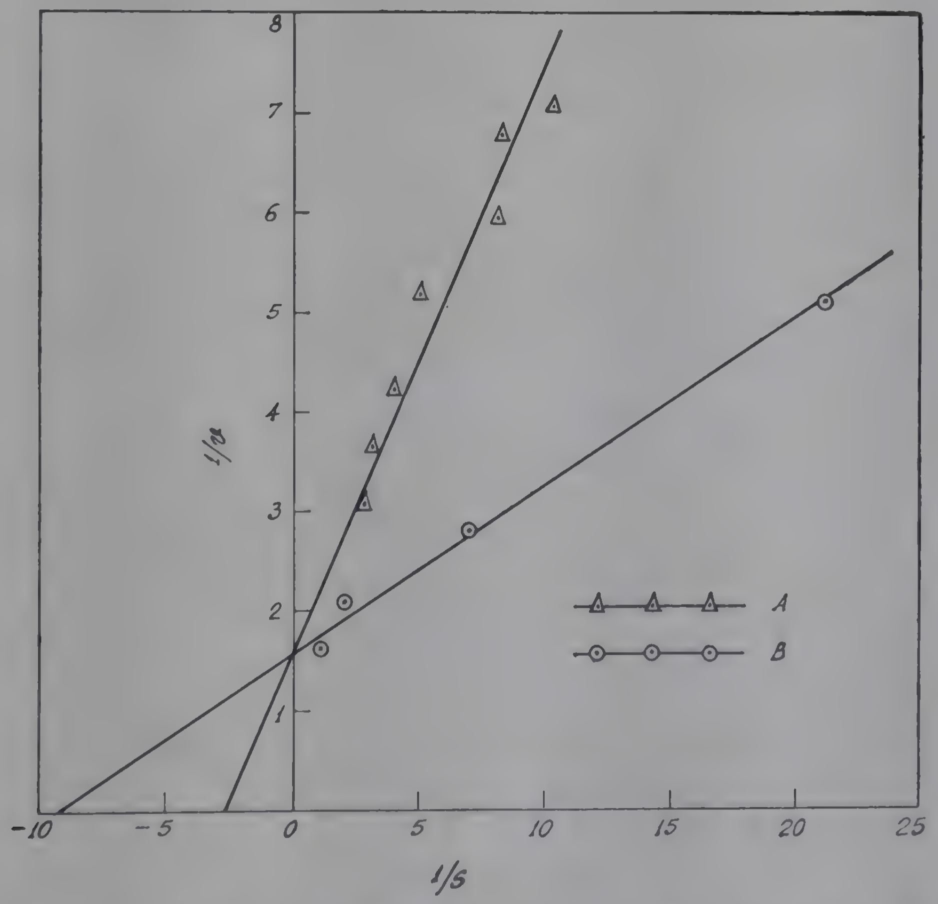

FIG. 3—KINETICS OF ATP AND GTP INCORPORATION: (A) K_s value $= 3{\cdot}7 \times 10^{-5} M$ when the primer is not added. (B) K_s value $= 1{\cdot}1 \times 10^{-5} M$ when the primer is added

TABLE 10—Nearest Neighbour Relationships After Incorporation of $AM^{32}PPP$

Dinucleotide sequence	Relative frequency	
	In absence of GTP	In presence of GTP
ApA	0·89	0·80
CpA	0·05	0·05
GpA	0·03	0·12
UpA	0·03	0·03

TABLE 11—Effect of Poly G on Glycine Incorporation

	c.p.m./mg. P
Complete system*	100
Complete system+poly G (0·1 ml. extract)	236
Complete system+poly G (0·2 ml. extract)	404

* Complete system contained the following: Tris (*p*H 7·2), 50 μmoles; $MgCl_2$, 3 μmoles; ATP, 2 μmoles; GTP, 1 μmole; PEP, 2 μmoles; glycine-^{14}C, 0·5 μmole of specific activity $1{\cdot}6\times10^6$ c.p.m./μmole; pyruvate kinase, 0·2 mg.; RNP particles, 3-4 mg.; *p*H 5 fraction, 1-2 mg.; and enzyme extract, 4 O.D. units ml.

position of its bases by two or three triplets of the same amino acid. That poly G may stand for glycine can be argued from doublet sharing code as well. Thus far, triplet code GUG, GAG and GCG has been assigned for glycine and GG may represent the doublet code[12]. Therefore, GGG should be equally effective for glycine besides GUG, GAG and GCG.

Discussion

Chloroplast grana seem to favour the incorporation of ATP and GTP into polynucleotide chains. This reaction is stimulated by Mn^{2+} ions. Poly A synthesis which is dependent on Mn^{2+} ions has recently been reported[13], but the ability to incorporate GTP is unique for this system. We have already reported that grana can synthesize poly A type and stroma poly U type of compound by which chloroplast can regulate amino acid incorporation in protein synthesis so far as phenylalanine is concerned. More data have now been obtained regarding lysine and proline. It seems that chloroplast constitutes a unique model by which it can regulate its amino acid incorporation into protein. The characteristic of this system which we are now reporting is that ATP incorporation is inhibited by

GTP in a competitive fashion. This may be interpreted in two ways: (1) that ATP and GTP are attached on the same chain making a co-polymer; or (2) ATP and GTP are incorporated in separate chains synthesizing two separate homopolymers either at the end of the acceptor RNA or separately without any acceptor RNA. Nearest neighbour frequency analysis supports the latter contention. The reaction seems to require a primer and the relative affinity for the substrates may be changed by limiting one primer or the other. That the product is either poly A or poly G type has been substantiated by its influence on amino acid incorporation; the triple G code has been assigned for glycine incorporation. What we have reported here suggests that there are several systems present in the chloroplasts for synthesizing polyribonucleotides, but in what way these polynucleotides are responsible for RNA synthesis in chloroplasts is yet to be answered.

SUMMARY

Chloroplast extract can catalyze the incorporation of four ribonucleoside triphosphates into polynucleotide chains. When chloroplasts are disrupted into stroma and grana, it is observed that the grana fraction favours the incorporation of ATP and GTP only and triphosphates are found to be the best substrates. Even after further purification it retains this activity. ATP incorporation is competitively inhibited by GTP and vice versa. The attachment of nucleoside triphosphates has been found mostly to be internal; only 5% of the incorporation is terminal. The *p*H optimum has been found to be around 8·5 and Mg^{2+} or Mn^{2+} is essential in different proportions for the two reactions. K_s value has been found to be around $1{\cdot}1 \times 10^{-5}$ *M*. The decrease in ATP incorporation after preincubation can be restored fully with added poly A and that of GTP by poly G. Nearest neighbour frequency analysis suggests that ATP and GTP are attached to two separate chains. That the product is poly A or poly G has been confirmed by its influence on amino acid incorporation by RNP particles; poly G enhances glycine incorporation. The implications of these findings have been discussed in relation to the structure of chloroplasts.

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Incorporation of Radioactive Nucleotides into Polyribonucleotides

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The experiments to be described in this paper have been carried out with the microorganism *Azotobacter vinelandii*. The senior author's experience with this microorganism (Strain 0, correctly referred to as *Azotobacter agilis*) dates back to 1955 in the laboratory of Prof. R. H. Burris at the University of Wisconsin, Madison, Wisconsin. You are all aware that it is a 'so called' autotrophic organism growing on one of the most simply defined medium composed of sucrose, phosphate, salt mixture and trace amounts of Fe and Mo. It does not require any nitrogen source as it can fix atmospheric nitrogen. Actually the primary object of the study in Dr Burris's laboratory was to throw light on the mechanism of nitrogen fixation. We were studying the kinetics of N_2 and NH_4^+ utilization to establish the key role of the latter in nitrogen fixation[1]. It appeared from the studies with the cell-free extracts of this microorganism that ^{15}N from $^{15}NH_4^+$ was very rapidly disseminated into various products like amino acids, purines, pyrimidines, etc.[2] Traces of ^{15}N could be detected in the protein as well. During the period 1954-55 studies on cell-free protein synthesis had just started in several laboratories. It was thought worthwhile to study the effect of various antibiotics and antimetabolites to study the inter-relationship between nucleic acid and protein syntheses in this microorganism. This work was carried out by Chakravorty and Burma[3] and further extended by Mandal[4] in our laboratories. We also described an amino acid incorporating system which comprised a comparatively heavy particulate fraction from this microorganism[5]. Though the free ribosomal particles have been known to be the major sites of protein synthesis in microorganisms, considerable evidence has also accumulated to demonstrate clearly that there could be other sites of protein synthesis as well. Association of ribosomes with the membrane material of the microorganisms clearly substantiates the capacity of these preparations for protein synthesis[6]. The involvement of RNA in protein synthesis was first demonstrated by Caspersson[7] and Brachet[8]. Their early cytological observations were strongly supported by biochemical evidence as the role of various types of nucleic acids in the different phases of protein synthesis became gradually clear.

The mechanism of duplication of DNA became understandable with the discovery of the enzyme DNA polymerase[9]. The mechanism of synthesis of RNAs, however, remained obscure for a while. The excitement that was associated with the discovery of the enzyme polynucleotide phosphorylase in *A. vinelandii* soon dwindled away as the physiological function of this enzyme in the synthesis of nucleic acids became gradually doubtful. So along with many other groups of workers, we in the laboratory of Prof. Severo Ochoa looked hard for an enzyme responsible for the synthesis of RNA. Our source material was the same microorganism, *A. vinelandii.* You are all familiar with the exciting history of the discovery of messenger RNA and the enzyme RNA polymerase responsible for the synthesis of messenger RNA. We reported the presence of this enzyme in two microorganisms, *A. vinelandii* and *Lactobacillus arabinosus*[10,11]. In sharp contrast to *A. vinelandii* enzyme, *L. arabinosus* polymerase was found to be Mn^{2+}-dependent instead of Mg^{2+}-dependent. The extensive purification of RNA polymerase from the extracts of *A. vinelandii* has since been reported by Krakow and Ochoa[12].

Sometime ago we were playing around with the extracts of *A. vinelandii* and to our utter surprise we found that this extract which is rich in polynucleotide phosphorylase activity was behaving differently (Fig. 1). Incorporation of ADP-8-^{14}C alone into RNA was much less than that in the presence of the other three nucleoside diphosphates. This is contrary to the expectation with polynucleotide phosphorylase which incorporates ADP-8-C^{14} alone at a much faster rate than in the presence of other diphosphates. This is due to more rapid formation of poly A than that of poly AGUC. Very soon we discovered the reason for this discrepancy. As soon as the extract was dialysed and phosphate was removed the behaviour of the extract was as expected for polynucleotide phosphorylase. So phosphate was making polynucleotide phosphorylase to behave in an unusual way (Fig. 2). This experience, however, taught us to guard against polynucleotide phosphorylase while searching for new enzymes responsible for the synthesis of RNA. As a routine check we add excess phosphate to the incubation mixtures and thus do not allow polynucleotide phosphorylase to catalyse any synthetic reaction. To do the preliminary experiments, however, we thought of using ^{32}P as tracer. Some of these results have already been described[13,14]. Extracts of *A. vinelandii* were found to catalyse the incorporation of ^{32}P into RNA-like material.

The incorporating system was dependent on Mg^{2+} and 3-PGA (or PEP). The 105,000 *g* particulate fraction as well as the 105,000 *g* supernatant

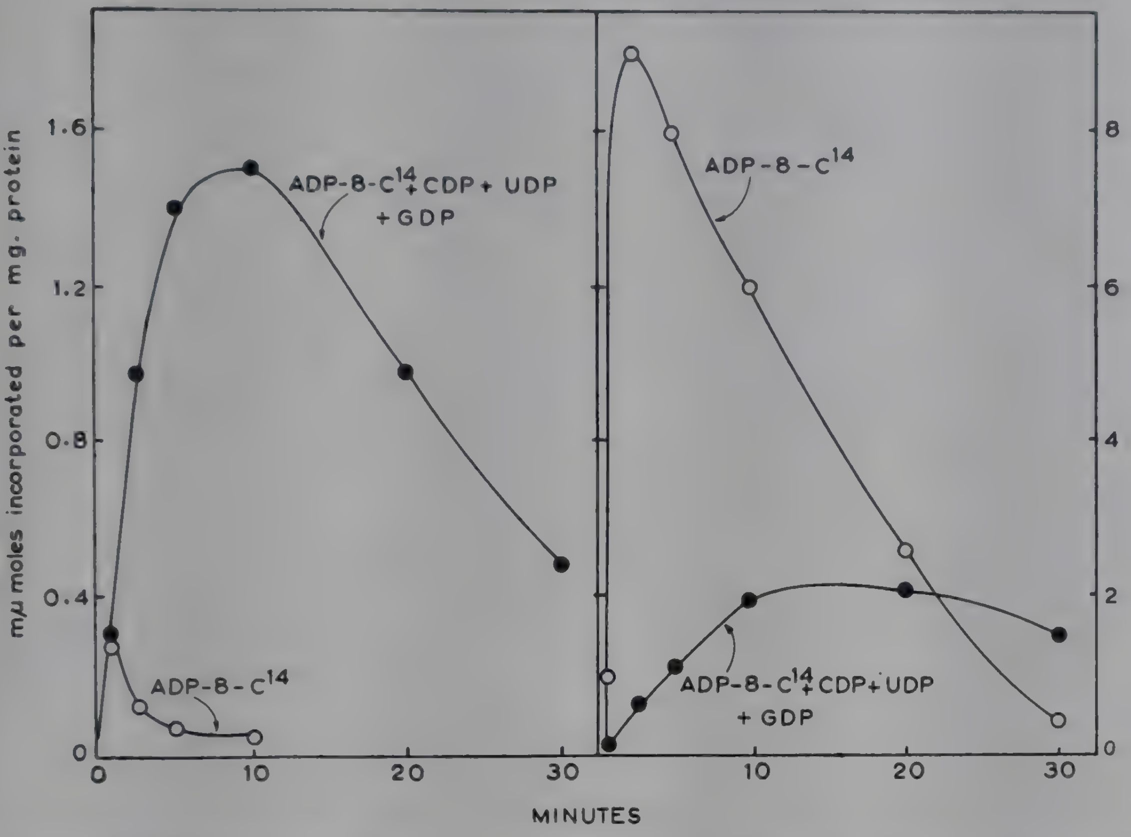

FIG. 1—INCORPORATION OF AMP-8-^{14}C FROM ADP-8-^{14}C INTO POLYRIBONUCLEOTIDE CHAIN IN THE PRESENCE AND THE ABSENCE OF OTHER NUCLEOSIDE TRIPHOSPHATES: Left figure—undialysed extract of *A. vinelandii*. Right figure—dialysed extract of *A. vinelandii*

fraction were essential for the incorporation. The properties of the system have been reported in detail elsewhere[14]. Since the extracts of *A. vinelandii* are rich in the enzyme polynucleotide phosphorylase, special care was taken to rule out the possibility that the phosphorylase is responsible for catalysing the incorporation of ^{32}P into RNA. The radioactive RNA isolated from the incubation mixture behaved as s-RNA on DEAE-cellulose column and all the four nucleotidic components were found to be radioactive[14].

Since we used ^{32}P as tracer at the inorganic phosphate level the pathway of incorporation of ^{32}P into RNA was naturally very much complicated. So we thought of using triphosphates to study the incorporation of ribonucleotides, if any, into RNA as in the case of DNA-dependent RNA polymerase system. One has, however, to guard against the possible attachment of the nucleotides at the end of the polyribonucleotide chains, specially of cytidylate and adenylate units at the end of s-RNA. Our initial criterion, however, was the stimulation of the incorporation of a single radioactive nucleotide in the presence of the other three nucleotides. Though that was not a very good criterion yet it helped to provide an assay method for the purification of the system. Some of the preliminary results obtained with an ammonium sulphate fraction are presented in Table 1. The incorporation of a single nucleotide like AMP from ATP-8-^{14}C is considerably enhanced in presence of other three nucleoside triphosphates, GTP, CTP and UTP. Omission of any one of them leads to diminished incorporation. It is obvious

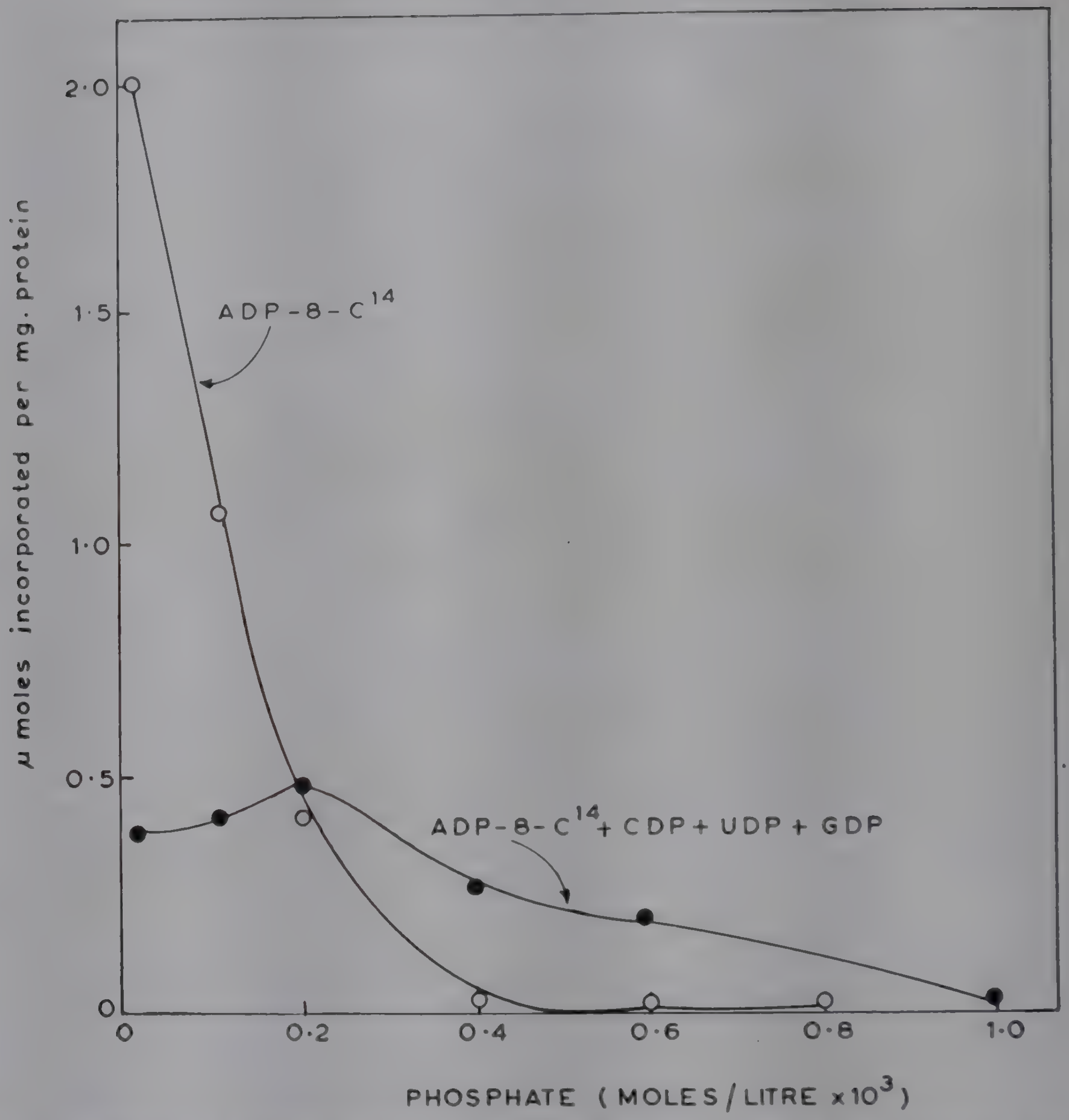

FIG. 2—EFFECT OF INORGANIC PHOSPHATE ON THE INCORPORATION OF AMP-8-^{14}C FROM ADP-8-^{14}C INTO POLYRIBONUCLEOTIDE CHAIN AS CATALYSED BY POLYNUCLEOTIDE PHOSPHORYLASE IN THE PRESENCE AND ABSENCE OF OTHER NUCLEOSIDE TRIPHOSPHATES[10]

TABLE 1—INCORPORATION OF AMP-8-^{14}C INTO RNA

Omission	Specific activity
—	0·81
CTP	0·31
GTP	0·31
UTP	0·34
UTP, GTP	0·28
CTP, UTP, GTP	0·22
Mg^{2+}	0

from these results that there is considerable amount of end attachment as well. Both the terminal and nonterminal additions are completely Mg^{2+}-dependent. Some of these results have already been published.

We thought of using this assay method, i.e. the incorporation of a single radioactive nucleotide in the presence of other three non-radioactive nucleoside triphosphates, for the purification of the enzyme, if there is any enzyme other than the end attaching enzyme or the DNA-dependent RNA polymerase. The purification procedure is described in Table 2. The steps are rather simple. However, 80% of the activity was lost in achieving a purification of only 20-22-fold and at the final stage we could not keep the enzyme fully active even for a day or two. The enzyme becomes very rapidly inactive. This has been our main handicap in the further purification of the enzyme. We tried DEAE-chromatography without success. We tried in vain to stabilize the enzyme in presence of various sulphydryl agents. We were forced to finish up any experiment we planned on the same day we prepared the enzyme. Anyway, we did a few convincing experiments, some of which have already been published[15].

The first experiment we tried with the partially purified enzyme fraction was to study the incorporation of GMP-8-^{14}C as it was expected not to attach itself to the terminus of s-RNA chain. Incidentally, in all these experiments we were trying s-RNA as primer. We were aware that the system was not completely free from DNA but at least we were not adding any extraneous DNA. There was considerable incorporation of GMP-8-^{14}C alone but the incorporation was stimulated about 3-fold by the addition of other three triphosphates[15]. Next we compared the incorporation of all the three nucleotides in the presence of others (Table 3). The rates of incorporation of AMP-8-^{14}C as well as UMP-2-^{14}C were more or less the same; the rate of incorporation of GMP-8-^{14}C was somewhat less. Incorporation of CMP was not tried. These results corroborate the fact that all the nucleotides are incorporated into RNA though it is difficult to decide whether the addition of each is terminal or non-terminal.

The next question we raised was whether the requirement for s-RNA was absolute (Table 4). We used s-RNA prepared from yeast by the phenolization method of Monier *et al.*[16] We analysed the RNA for the contaminants DNA. The amount was found to be trace and could not account for the incorporation rates observed. In control experiments addition of similar amount of DNA did not result in any significant incorporation of the nucleotides. Omission of s-RNA led to marked reduction in the incorporation rate. When yeast s-RNA was replaced by *A. vinelandii* s-RNA, the incorporation occurred to about the same extent. Use of ribosomal RNA instead of s-RNA leads to diminished incorporation. We are not sure, however, whether our ribosomal preparation was contaminated with s-RNA. It is planned to use various types of RNA to decide the issue whether s-RNA alone serves as the primer. The dependency on s-RNA is shown

TABLE 2—PARTIAL PURIFICATION OF s-RNA-DEPENDENT ACTIVITY

Fraction	Total units	Specific activity
Crude extract	250	0·27
Ammonium sulphate I (35-55)	300	0·70
Protamine precipitate	184	1·25
Ammonium sulphate II (55-90)	35	5·83

TABLE 3—INCORPORATION OF RADIOACTIVE RIBONUCLEOTIDES

Nucleoside triphosphates	mμmole incorporated/mg. protein
ATP+8-^{14}C+GTP+UTP+CTP	4·8
UTP+2-^{14}C+ATP+GTP+CTP	5·0
GTP-8-^{14}C+ATP+UTP+CTP	4·0

TABLE 4—DEPENDENCY OF THE INCORPORATING SYSTEM ON s-RNA
(Majumdar and Burma[15])

RNA	mμmole of AMP-8-^{14}C incorporated/mg. protein
None	0·5
Yeast s-RNA	5·8
A. vinelandii s-RNA	6·5
A. vinelandii ribosomal RNA	2·5

more clearly in Fig. 3. The rate of incorporation increases linearly with the increasing amount of s-RNA added. Further, increase in s-RNA content (above 70μg.) leads to inhibition of the incorporation process. Addition of poly A to the incubation mixture does not stimulate the incorporation to any measurable extent.

We were aware that the partially purified fraction which was used in these experiments was still contaminated with RNA polymerase activity. The rate of incorporation of a nucleotide in the presence of DNA was about one-third of that in the presence of s-RNA. So we thought of using the

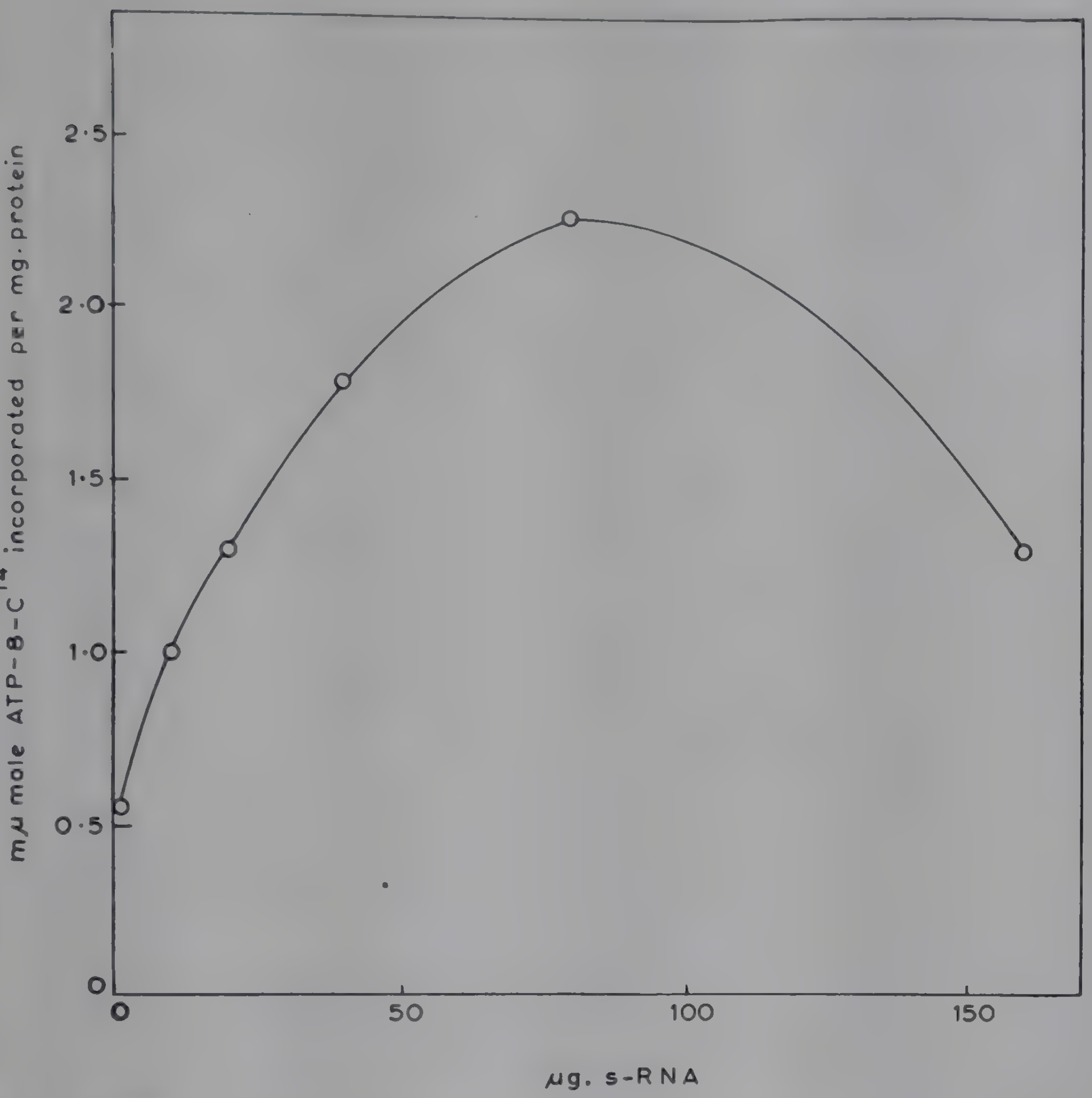

Fig. 3—Effect of varying concentrations of s-RNA on the rate of incorporation of AMP-8-^{14}C from ATP-8-^{14}C into polyribonucleotide chain in the presence of other nucleoside triphosphates

different ammonium sulphate fractions to compare the s-RNA and DNA-dependent activities. The results are presented in Table 5. The ratio of the two activities varies from 0·7 to about 3 indicating that the two activities are different. This was also supported by the effect of the nucleases on the incorporation rates. The results are given in Table 6. The effect of DNAase on the incorporation is very little in comparison with that of RNAase. RNAase as expected completely abolishes the incorporation. The involvement of RNA instead of DNA was also supported by the effect of actinomycin D (Table 7) which is known to interfere with DNA-primed RNA polymerase action. Ammonium sulphate fraction (30-55%) which had most of the DNA-dependent RNA-polymerase activity was found to be sensitive to actinomycin D (4μg./ml.). The rate of incorporation was diminished to the extent of 50 %. The incorporation in the presence of s-RNA as catalysed by the ammonium sulphate (55-90%) fraction remained unaffected in the presence of actinomycin D.

TABLE 5—VARIATION IN THE RATIO OF THE NUCLEOTIDE INCORPORATING ACTIVITIES WITH s-RNA AND DNA AS PRIMERS

(Majumdar and Burma[15])

Enzymic fraction	Primer	mμmoles of AMP-8-^{14}C incorporated/ mg. protein	Ratio (s-RNA/DNA)
Ammonium sulphate (0-30%)	s-RNA	2·0	0·67
	DNA	3·0	
Ammonium sulphate (30-55%)	s-RNA	1·7	0·15
	DNA	11·3	
Ammonium sulphate (55-90%)	s-RNA	5·5	2·75
	DNA	2·0	

TABLE 6—EFFECT OF NUCLEASES ON THE INCORPORATION OF AMP-8-^{14}C

Treatment	Specific activity
—	0·77
DNAase	0·59
RNAase	0·03

TABLE 7—SENSITIVITIES OF THE DNA-DEPENDENT AND s-RNA-DEPENDENT ACTIVITIES TO ACTINOMYCIN D

(Majumdar and Burma[15])

Enzyme fraction	Primer	Actinomycin D	mμmole of AMP-8-^{14}C incorporated/ mg. protein
Ammonium sulphate (30-55%)	DNA	—	10·6
		+	5·0
Ammonium sulphate (55-90%)	s-RNA	—	5·6
		+	6·3

TABLE 8—DISTRIBUTION OF RADIOACTIVITY IN RNA

	c.p.m.	% of total
Adenosine	46	12
Adenylic acid	329	88

The major question is, however, whether this type of attachment is terminal or non-terminal. The answer is not quite satisfactory. In one set of preliminary experiments (Table 8) we tried to decide whether incorporated adenine was released as riboside or ribotide by alkali treatment. Radioactive RNA was isolated by the phenol method from the incubation mixture in which ATP-8-^{14}C was used as the radioactive marker. The isolated RNA was degraded with alkali and after neutralization with $HClO_4$, the hydrolysate was subjected to electrophoresis. Considerable amount of radioactivity (about 12%) was recovered as adenosine indicating extensive end attachment, though major amount was recovered as adenylic acid due to non-terminal attachment. Similar experiments have been carried out with ATP-*a*-^{32}P as marker nucleotide and all the nucleotides were found to be radioactive. This, however, does not rule out the possibility that AMP may be attached to the terminus of several types of s-RNA ending in various types of bases. Experiments with GTP-*a*-^{32}P as well as studies on the behaviour of the newly synthesized RNA to the action of phosphodiesterases are in progress. On completion of these studies we will be able to tell whether the attachment is mostly terminal or at least partly non-terminal.

In the meantime we decided to test whether the amino acid accepting capacity of the s-RNA used as a primer is affected anyway by incubation in the presence of all the four nucleoside triphosphates and the *A. vinelandii* enzyme. For this we used excess of *p*H 5 fraction prepared from rat liver and used several amino acids (glycine, leucine, phenylalanine, serine and histidine) for loading. The complete system contained s-RNA, the amino acids, all the four nucleoside triphosphates and excess of *p*H 5 fraction. It was expected that any repairing of s-RNA by the end attachment of CMP and AMP residues would take place under these conditions and maximum loading of s-RNA will be displayed. On the top of this incubation mixture *A. vinelandii* enzyme fraction was added which was expected to incorporate all the four nucleoside triphosphates. There was small but significant increase in the loading capacity of s-RNA (Table 9). This might, however, indicate a somewhat more extensive repairing of s-RNA as observed by Canellakis[17] after treatment of s-RNA with phosphodiesterase.

Before concluding, I will describe the property of another fraction which we obtained during the partial purification of s-RNA-dependent activity. During the second ammonium sulphate fractionation the protein precipitating between saturation 0-30 was found to incorporate GMP-8-^{14}C at almost

TABLE 9—INCREASE IN AMINO ACID ACCEPTING CAPACITY

Incubation mixture	c.p.m.	% increase
Complete system	1048	—
Complete system + *A. vinelandii* fraction	1317	30

5 times the rate of AMP-8-^{14}C. In the beginning we took this as an error of measurement. We were actually measuring the RNA polymerase activity and so had the DNA prepared from calf thymus always present in the incubation mixture. All the four nucleoside triphosphates were naturally added (Table 10). On omission of the triphosphates in the incubation mixture the rate of incorporation was diminished only to a very small extent. This roused the suspicion that perhaps we were synthesizing poly G type of material, but the data are in too premature a state to arrive at any definite conclusion. We are, however, convinced that DNA is actually involved in the enhanced incorporation of GMP-8-^{14}C alone (Fig. 4). Increase in the amount of DNA leads to enhanced incorporation until saturation limit is attained with approximately 100μg. of DNA. But to our great surprise the system is found to be insensitive to actinomycin D (Table 10). Actinomycin D is known to bind specifically with the guanine residues in DNA. In this case, however, addition of guanylate units may be at the end of DNA chain as observed by Canellakis and his coworkers[17,18]. Only detailed experimentation will provide the final answer.

Various groups of workers have reported various types of incorporation of ribonucleotides[19–23]. Of these, the most interesting are those reported by Canellakis and his coworkers[17,18] who demonstrated the attachment of the ribonucleotides at the end of DNA chain. Formation of homo- and copolymers of ribonucleotides was known since the discovery of the enzyme polynucleotide phosphorylase. Similar reactions, like the formation of poly A from ATP instead of ADP, have been observed in many other systems[23–27]. These reactions mimic the RNA polymerase-catalysed reaction. In this Symposium you will listen to several types of similar reactions. The physiological importance of these reactions are not yet very clear. DNA polymerase is definitely known to catalyse the formation of homo- and copolymers under certain conditions. Same is true for RNA polymerase-catalysed reaction[25]. There is, however, enough evidence in the literature to indicate that there may be enzymes other than RNA polymerase which can catalyse the formation of homo- and copolymers[23,24,26,27]. Further, besides attaching the ribonucleotides at the end of s-RNA chain, some of them can extensively incorporate ribonucleotides into the interior of the polyribonucleotide

TABLE 10—INCORPORATION OF GMP-8-^{14}C AND AMP-8-^{14}C

Nucleoside triphosphates	mμmole incorporated/mg. protein
GTP-8-^{14}C	16·4
GTP-8-^{14}C+ATP+UTP+CTP	22·8
ATP-8-^{14}C+UTP+CTP+GTP	4·0
GTP-8-^{14}C+UTP+CTP+ATP+actinomycin D	16·0

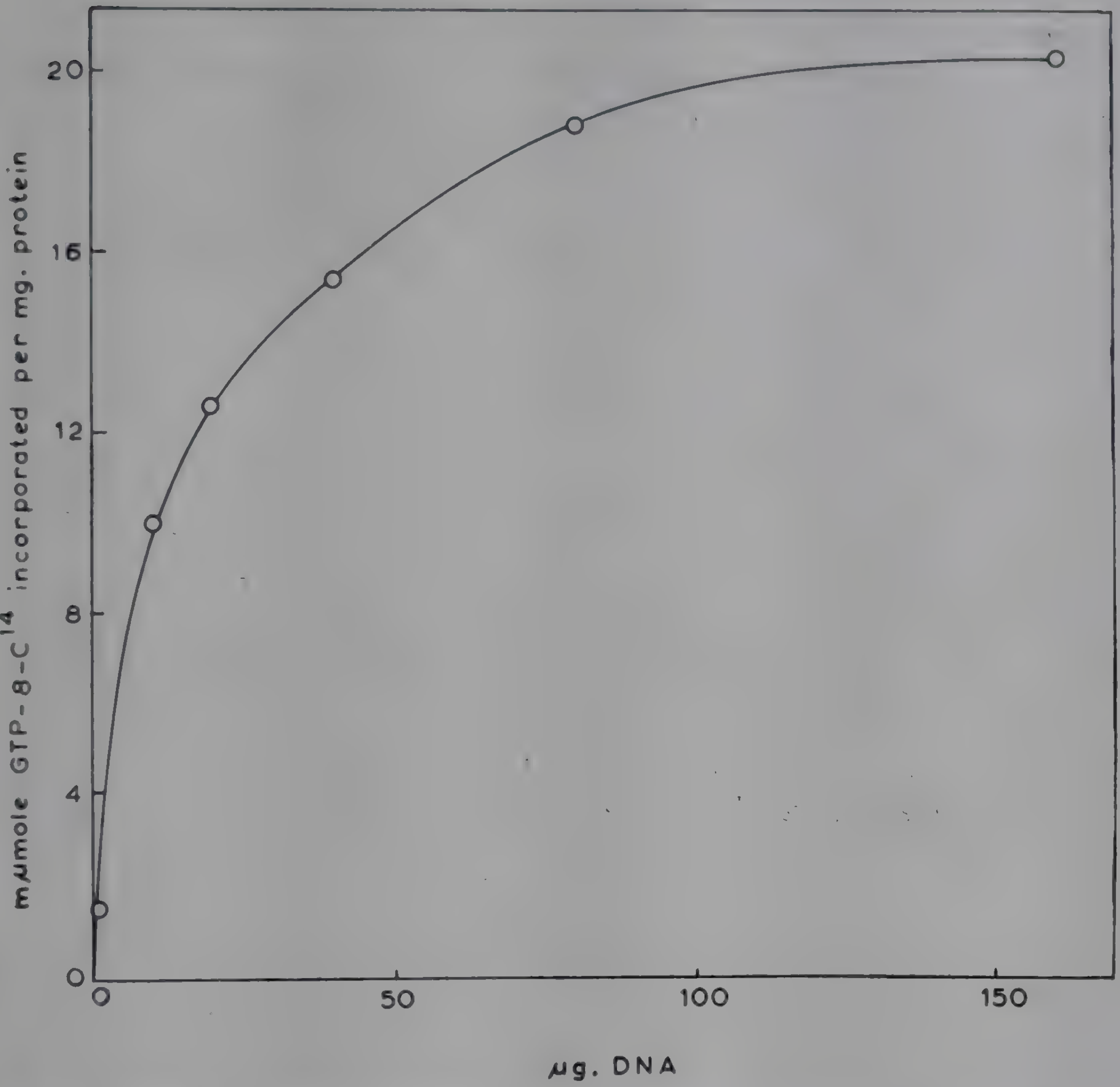

Fig. 4—Effect of varying concentrations of DNA on the rate of incorporation of GMP-8-^{14}C from GTP-8-^{14}C into polyribonucleotide chain

chain, e.g. ribosomal RNA[20]. Are all these reactions as observed in test tubes artifact reactions or have they any significance in terms of physiological function? This question remains practically unanswered.

When we first got the indication of non-terminal incorporation of ribonucleotides into s-RNA we thought that this reaction represented the synthesis of s-RNA. Subsequently, however, evidences were produced by many through the hybridization experiments[28,29], the results obtained with the nuclei[30] and the experiments with the use of actinomycin D[31], in favour of the fact that total cellular RNA synthesis is DNA-dependent. The original suggestion of McCully and Cantoni[32] that the synthesis of s-RNA is self-dependent is not very much favoured now. Even though the evidence in favour of actinomycin D insensitive synthesis of RNA is not quite rare[33] and the direct formation of s-RNA on DNA template is yet to be demonstrated, it appears in all probability that the coding for s-RNA molecules resides on the DNA genome. The only enzyme which is known to use RNA as template for duplication of RNA is the one which is induced in the host cell by the RNA-containing viruses[34,35]. It may not, however, be unnatural to believe with the existing data that the type of reaction observed by us may represent limited synthesis of s-RNA which may mean either the minor repair of the damages near the ends or even of the breaks and deletions in the interior of the chain. Or, it could be that the partial reactions which we are observing are due to the alteration produced in RNA polymerase during the purification procedure. Somewhat altered active sites may result in these artificial reactions.

Acknowledgement

Sincerest thanks are due to Dr D. M. Bose, Director, Bose Institute, for his kind interest and continued encouragement. The financial assistance of the Rockefeller Foundation, U.S.A., the Council of Scientific & Industrial Research, India, and the Indian Council of Medical Research, India, are also gratefully acknowledged. Thanks are also due to Elsevier Publishing Company, Amsterdam, The Netherlands and the National Academy of Sciences, Washington, D.C., U.S.A. for the kind permission of reproduction of some of the results already published in *Biochimica Biophysica Acta* and *Proceeding of the National Academy of Sciences*, *Washington* respectively.

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Nuclear Synthesis of RNA in *B. megaterium* and the Effects of Histones on RNA Synthesis

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The organism *B. megaterium* has proved to be a convenient one for studying the interactions of the various parts of the cell in relation to nucleic acid and protein synthesis. The protoplast is capable of most of the synthetic processes which occur in the intact cell, e.g. DNA, RNA and protein synthesis and the reactions providing their precursors. It is also easily fractionated, after disrupting the protoplast membrane with a non-ionic detergent such as lubrol into a number of fractions.

On centrifuging the lysed protoplast a nuclear fraction is obtained[1] which contains nearly all the DNA of the cell and 12-15% of the RNA, together with some protein and membranes. The greater part of the RNA is present as ribosomes. When this fraction is resuspended in a buffer solution and again centrifuged, the membranes are sedimented and the ribosomes and the DNA containing material remain in the supernatant. By appropriate sedimentation the ribosomes, which have been termed the 'nuclear ribosomes' and the DNA containing material, termed the nuclear material, can be separated. The latter contains in addition to DNA only 2-4% of the RNA of the cell. The supernatant 'cytoplasmic fraction', which remains after the first sedimentation contains the cytoplasmic ribosomes and soluble RNA, which can be separated by the usual procedures. This process is shown in Chart 1.

The biosynthesis and subsequent history of the RNA have been studied[2] by incubating protoplasts for various times with a medium containing tritiated uridine, and then preparing the various fractions. Some results are given in Fig. 1.

It can be seen that for short times of incubation the 'nuclear' fractions are much more highly labelled than the cytoplasmic fractions. In only two minutes the activity in the "nuclear material" has reached 3/4 of the value

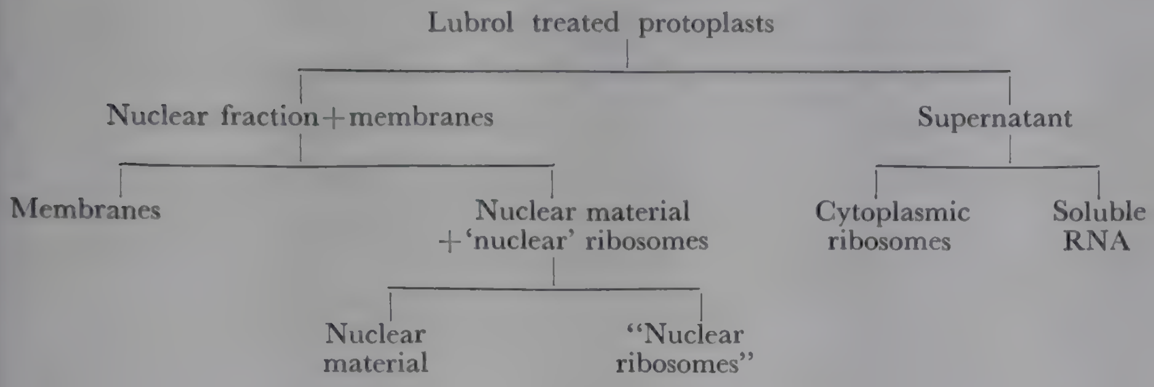
Lubrol treated protoplasts
Nuclear fraction+membranes
Supernatant
Membranes
Nuclear material
+'nuclear' ribosomes
Cytoplasmic
ribosomes
Soluble
RNA
Nuclear
material
"Nuclear
ribosomes"

CHART 1

it reaches after 20 min. There is thus an extremely high rate of labelling of the RNA in this fraction. Sedimentation experiments in caesium chloride gradients showed that this RNA was firmly bound to the DNA, probably as a hybrid or part hybrid and is not removed by RNAase. On account of this very high rate of turnover this may be regarded as the site of synthesis of much of the RNA of the protoplast.

This has been confirmed by the observation[3] that the nuclear fraction is by itself capable of RNA synthesis when incubated with suitable precursors and that the activity initially associated with the DNA can be transferred to ribosomes.

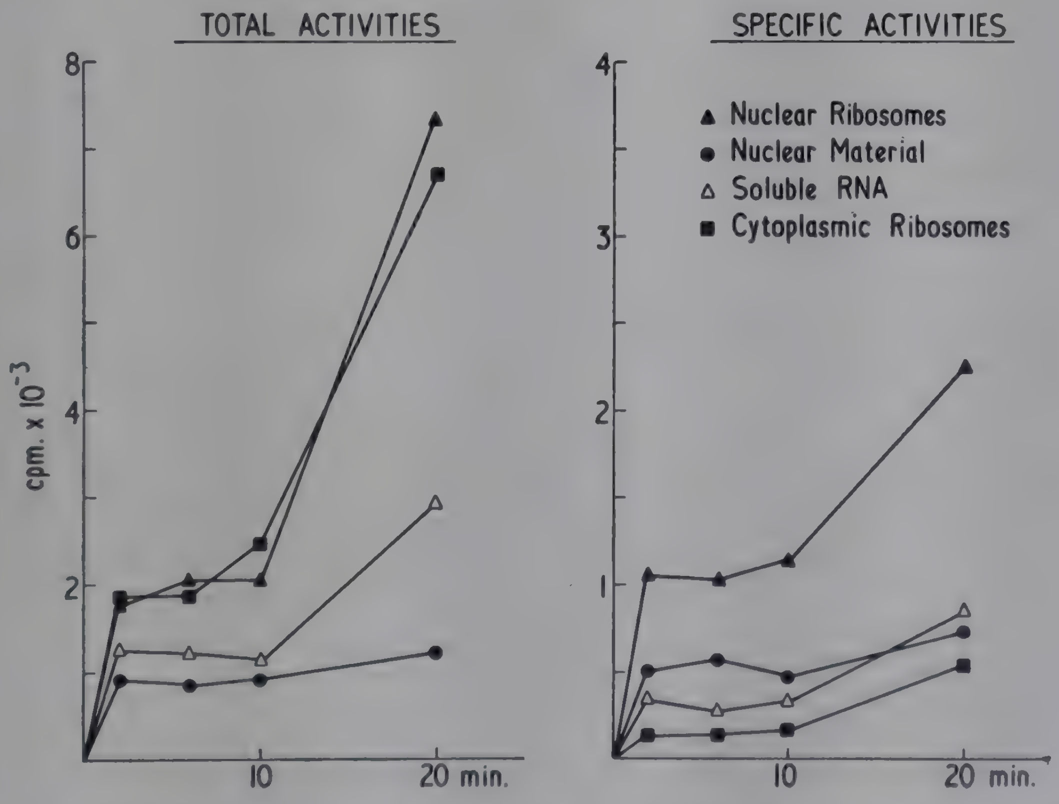

FIG. 1—BIOSYNTHESIS OF RNA IN PROTOPLASTS OF *B. megaterium* INCUBATED WITH MEDIUM CONTAINING TRITIATED URIDINE FOR VARIOUS TIMES (from G. N. Godson & J.A.V. Butler)

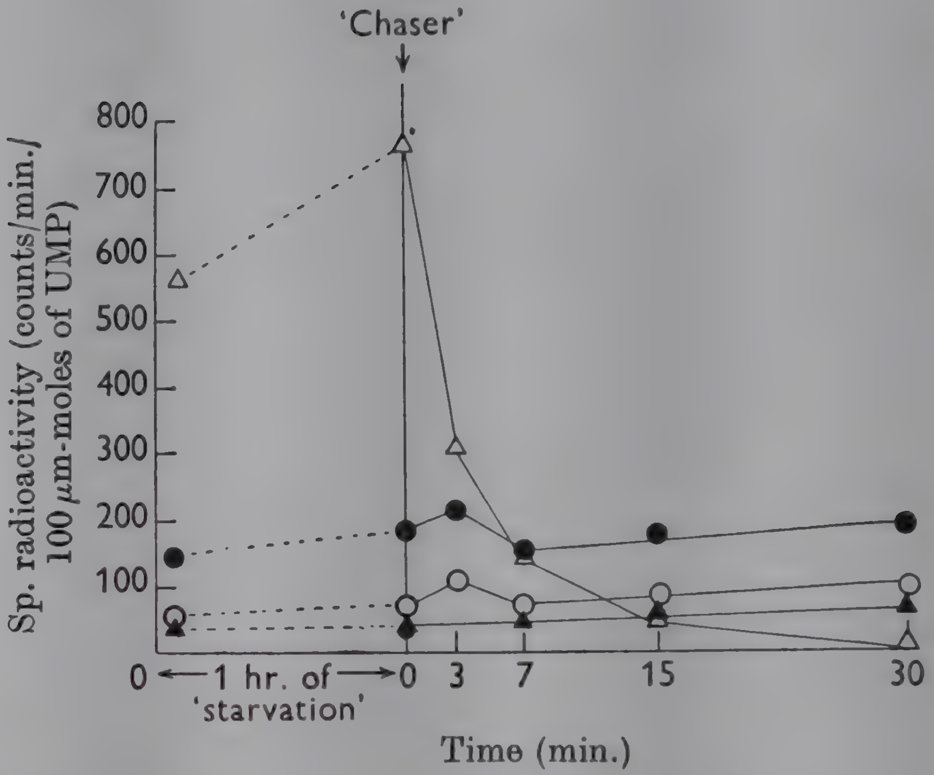

Fig. 2—"Chasing experiments" with *B. megaterium* protoplasts labelled with ^{3}H-uridine and then starved for 1 hr, followed by "chase" by addition of an excess of inactive uridine: Reproduced from *Biochem. J.*, ref. 2; △ Nuclear material; ○ Soluble RNA; ▲ Cytoplasmic ribosomes; ● Nuclear ribosomes

A high turnover rate of the RNA in this fraction has also been indicated by "chasing" experiments[2] on protoplasts which have been labelled and then starved for 1 hr to reduce the radioactive precursor pool, before adding non-radioactive precursor as chaser (Fig. 2). The radioactivity in the nuclear fraction is reduced to half in a few minutes although the time varies from experiment to experiment.

This work, therefore, demonstrates the initial synthesis of RNA in association with the DNA in the nuclear material followed by its transfer to the ribosomal fractions, the 'nuclear' ribosomes being labelled more rapidly than the 'cytoplasmic' ribosomes (Fig. 1). The reason for this must now be discussed. There is some doubt as to the origin of the 'nuclear' ribosomes. They are sedimented in the original centrifugation, in the fraction which contains both the nuclear material and the membranes. When this fraction is resuspended and centrifuged these ribosomes remain in the supernatant with the nuclear material. It is thus possible that they were initially associated with the membrane part of this fraction. However, if it is assumed that the RNA is synthesized in the nuclear material, and the high radioactivity associated with these ribosomes is representative of newly synthesized RNA, these ribosomes must also have been initially closely associated with the nuclear material. The designation "nuclear ribosomes" is thus justified.

We have then to ask if the 'nuclear' ribosomes differ from those in the cytoplasm in any important respect, other than the high degree of labelling. Both types of ribosomes have been examined by sucrose gradient centrifugation at both high ($10^{-2}M$) and low ($10^{-4}M$) magnesium ion concentrations. Some typical results are shown in Fig. 3. The behaviour is similar to that

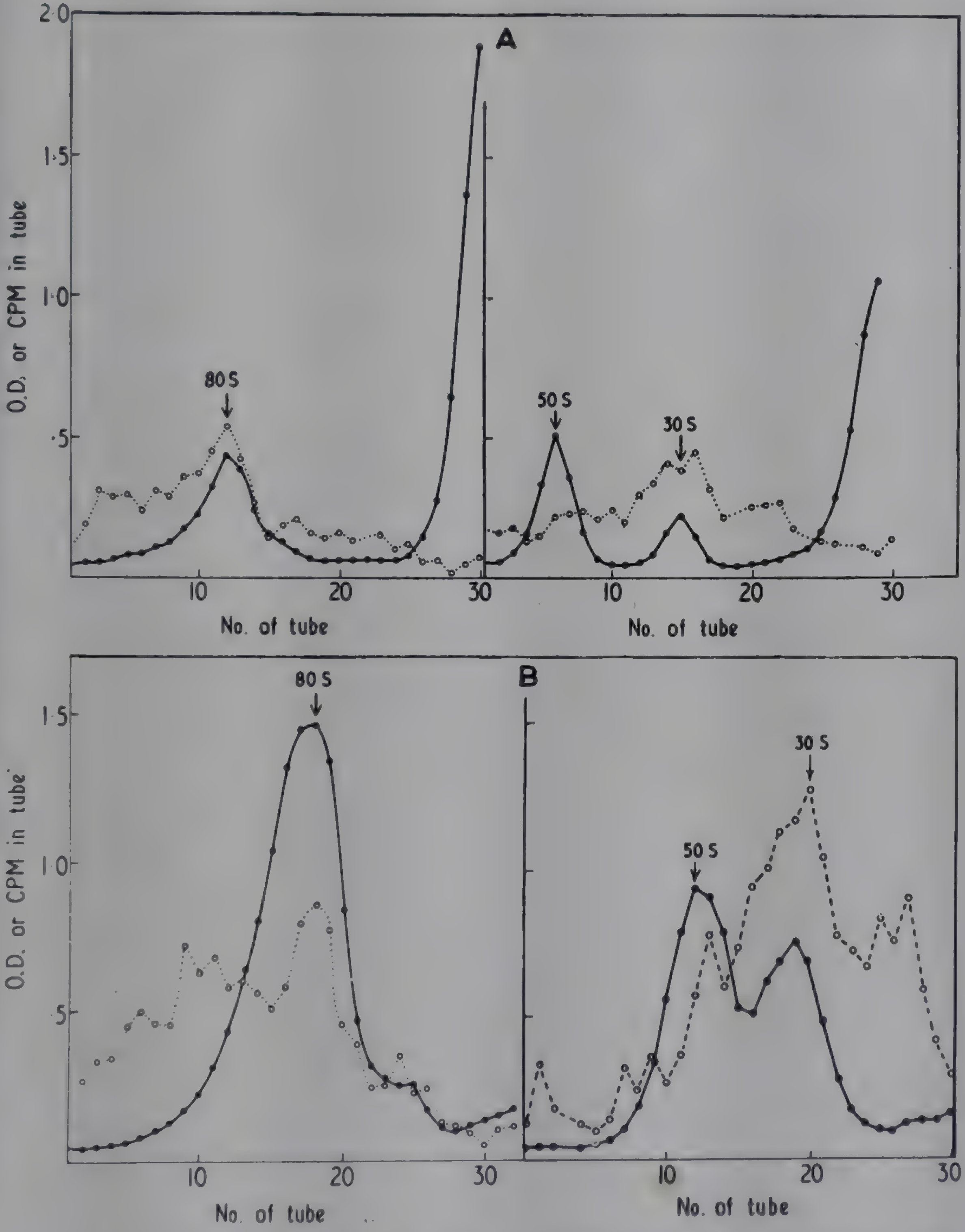

FIG. 3—SUCROSE GRADIENT SEDIMENTATION PATTERNS OF LABELLED RIBOSOMES FROM (A) NUCLEAR, (B) CYTOPLASMIC RIBOSOMES: Diagrams on left in medium containing $10^{-2}M$ magnesium chloride; on right in medium containing $10^{-4}M$ magnesium chloride. Incubation period in all cases, 15 min.

which has been observed with *Esch. coli*[4]. It can be seen that in $10^{-2}\,M$ magnesium ions, both types give the well-known single peak[5,6] of optical density at 70-100 S, while the radioactivity (newly formed RNA) is spread over a broad band, partly coincident with the optical band and partly at the higher S values (>100 S), which have been attributed to "active ribosomes" (Riseborough *et al.*)[5,6] or to ribosomal aggregates (Gierer[7] and Warner *et al.*[8] The relative amount of label in the 80 S peak at different times in ribosomes of the two kinds is shown in Fig. 4. It is found that the specific activity in this peak in the nuclear ribosomes is 2-5 times that of the cytoplasmic.

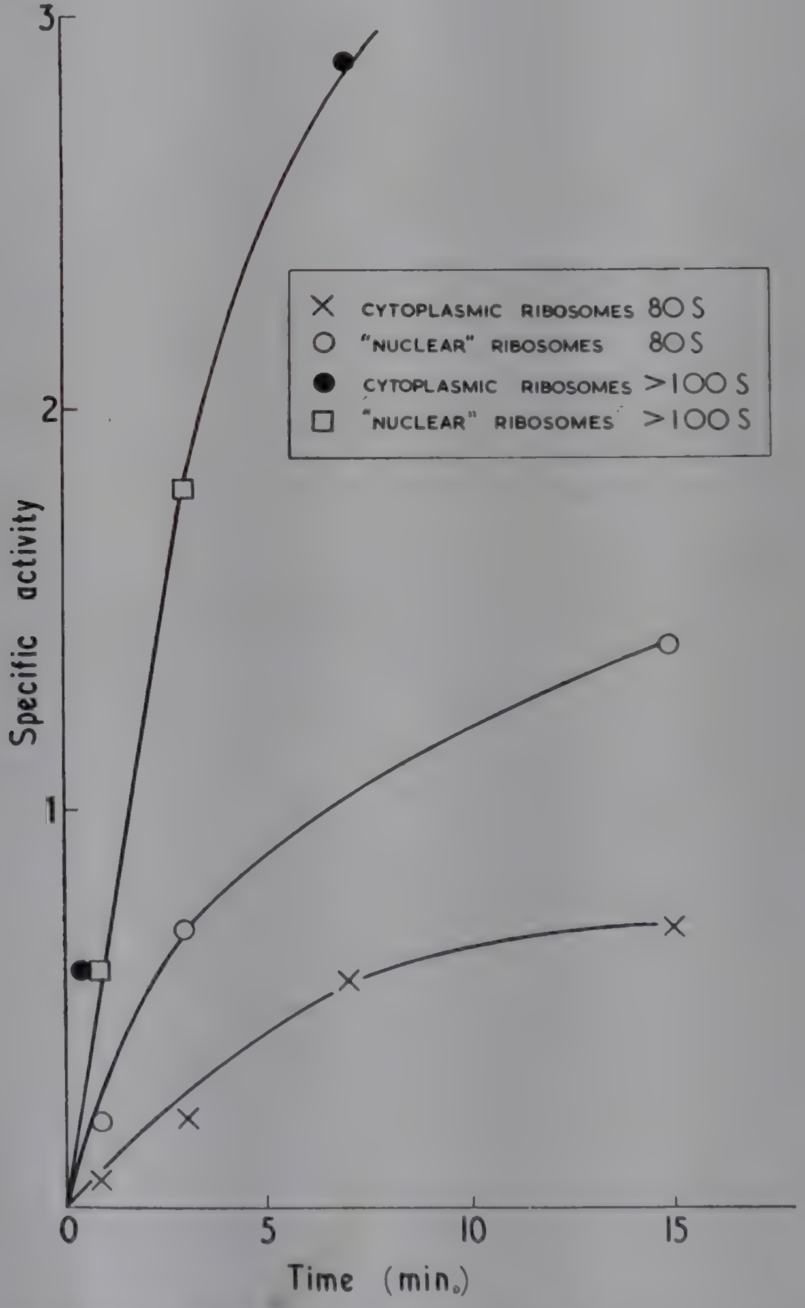

FIG. 4—RELATIVE SPECIFIC ACTIVITIES OF ^{3}H-URIDINE IN 80 S PEAK OF 'NUCLEAR' AND CYTOPLASMIC RIBOSOMES, AFTER DIFFERENT TIMES OF INCUBATION: The specific activities in ribosomes sedimenting > 100S are also shown

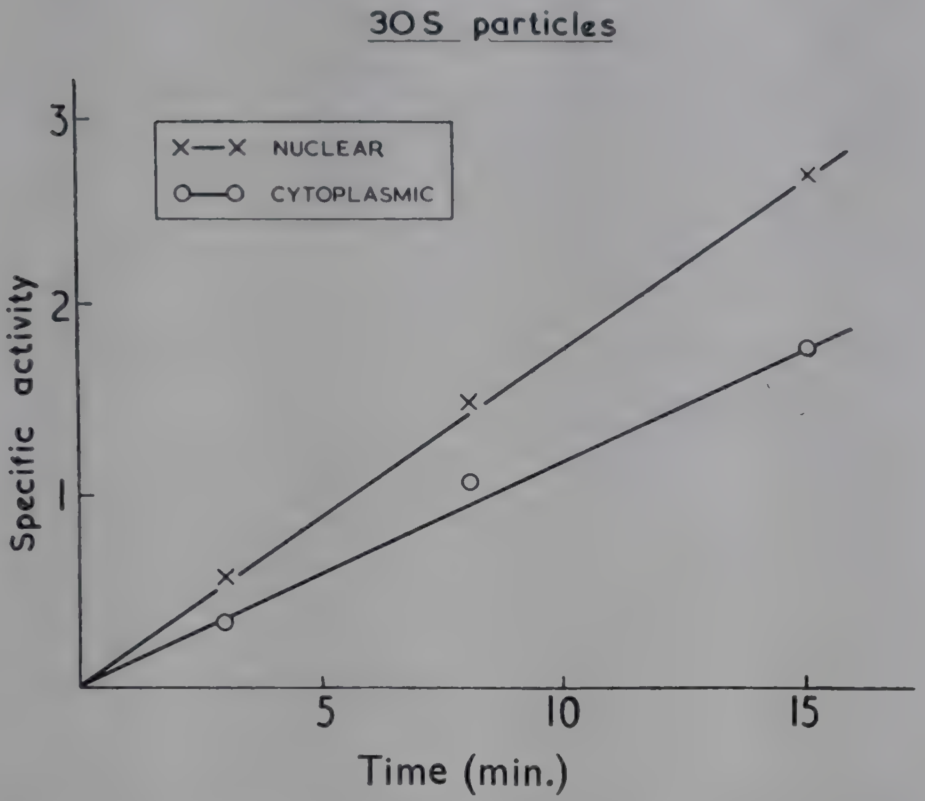

FIG. 5—RELATIVE SPECIFIC ACTIVITIES OF ^{3}H-URIDINE IN 30 S PEAK OF "NUCLEAR" AND CYTOPLASMIC RIBOSOMES

In $10^{-4}M$ magnesium, the 70 S peak splits into two peaks at about 50 S and 30 S as has been observed in other cases[5–8]. Much of the radioactivity previously present at high values of S is now displaced to values of $S < 30$, but some activity remains in the 30 S peak and to a less extent in the 50 S peak (Fig. 5). This is the case with both types of ribosomes. The radioactivity in the 30 S peak of the nuclear ribosomes is higher than that of the cytoplasmic, but the difference is not so great as in the 80 S peak.

In these experiments the 'nuclear' and cytoplasmic ribosomes do not appear to differ in any important way, except the higher degree of labelling of the former. This must arise from their closer association with the nuclear material containing DNA, where the new RNA presumably originates. This is confirmed by an experiment with actinomycin D, which inhibits synthesis of RNA on the DNA template[9,10]. Table 1 shows that the activity of the RNA in both nuclear and cytoplasmic ribosomes as well as that of the membrane and nuclear material is greatly reduced by this treatment while the labelling of soluble RNA is not affected.

TABLE 1—RNA SYNTHESIS IN PROTOPLASTS INCUBATED FOR 8 MIN. WITH AND WITHOUT ACTINOMYCIN D
(c.p.m./mg. RNA)

	Nuclear ribosomes	Cytoplasmic ribosomes	Soluble RNA	Nuclear material	Membranes
Actinomycin—	2700	485	835	5500	6500
Actinomycin+	95	24	770	760	408

We must suppose that the 'nuclear' ribosomes receive their label while associated with DNA and remain attached to it or to the membranes to a sufficient extent to be sedimented with them. It is quite possible that there is a direct contact between the nuclear material and membranes which permits the newly formed messenger RNA to pass from it directly to ribosomes attached to membranes in some way and that only later does release into the cytoplasmic fraction occur. It was shown by Godson, Hunter and Butler[11] that the crude membrane fraction containing membranes as well as ribosomes was very active in protein synthesis and Schlessinger[12] has recently made a similar observation, with the additional finding that the ribosomes are partially present as polyribosomes. A highly active RNA tightly bound to the lipoprotein membrane is also present (Table 1). The lipoprotein membrane might well be associated with the mechanism for removing the newly formed messenger RNA molecules from the DNA.

We can now consider some features of the labelling of the 30 S and 50 S bands themselves, which is shown in the sedimentation diagrams. Is this label due to newly synthesized ribosomal RNA or to attached messenger which has not been removed at the low magnesium content? One test of this which may be applied is to see whether the specific activity is constant throughout each band. If the labelling is ribosomal, the specific activity throughout the band should be reasonably constant, i.e. the curve of c.p.m. will have the same shape as that of the optical density. If the labelling is not ribosomal the activity may be concentrated in some part of the band. When this test is applied it is seen that the uniformity of label holds better for the 30 S band than for the 50 S band. However, the specific activity in the 30 S peak does increase along its heavier side and the region of high specific activity extends through the trough between the peaks into the 80 S peak (Fig. 6). Indeed in some sedimentation diagrams the radioactivity peak associated with the 30 S band seems to extend well into the region of the 50 S band. A possible interpretation of this is that some of the 30 S particles in $10^{-4}M$ magnesium are associated with messenger RNA, which gives them some higher S values, extending into part of the 50 S band.

Two other characteristic features of messenger RNA, which have been used as criteria are: (1) it is almost completely dissociated from ribosomes at low magnesium ion concentrations[13]; and (2) it is metabolically unstable, having a half life in bacteria of approximately 2 min.[14] To check the possibility that the magnesium ion concentration used above was not low enough, the experiment was repeated in $10^{-6}M$ magnesium but no changes in the activity of the 30 S peak were observed. Also when the ribosomes were washed with 0·1% deoxycholate the activity was not removed from this peak and its vicinity.

To test the metabolic stability of the RNA, protoplasts were labelled with tritiated uridine for a short period and either treated with actinomycin D to arrest RNA synthesis or "chased" with unlabelled uridine. Both these

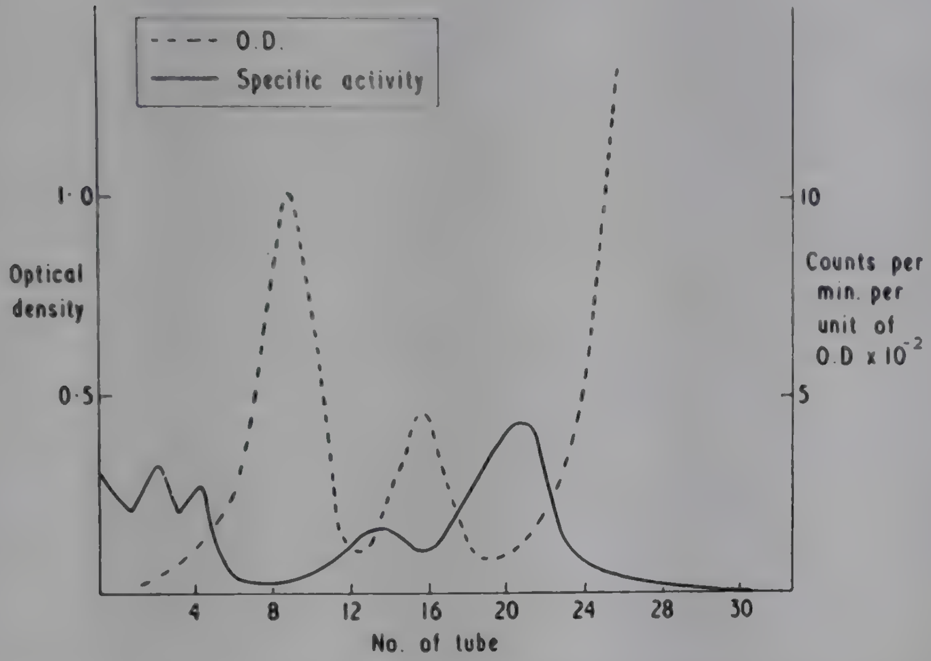

FIG. 6—SPECIFIC ACTIVITIES IN RNA THROUGHOUT THE SUCROSE GRADIENT SEDIMENTATION DIAGRAM OF NUCLEAR RIBOSOMES LABELLED FOR 8 MIN. IN INCUBATION MEDIUM CONTAINING ^{3}H-URIDINE

treatments were continued for 10 min. so that any metabolically labelled RNA would be lost. In both cases there was no loss of label in this region. Therefore, if this activity is messenger, it must be of a fairly stable nature.

When RNA is extracted from labelled protoplasts or from the sedimented nuclear or cytoplasmic ribosomes and examined on sucrose gradients, a peak of activity associated with the 16 S peak is observed, in addition to degraded messenger at low values of S, with very little activity in the 23 S peak.

The label associated with the 30 S ribosomal peak is thus (1) tightly bound and (2) metabolically stable. These are not the characteristics previously observed of messenger RNA.

Our conclusion is that the nuclear ribosomes are preferentially labelled due to the close association of these ribosomes with the DNA-containing material at the time of synthesis of RNA. This suggests as a possibility that ribosomes from the cytoplasm assist actively in removing the molecules of messenger RNA from the template on which they are formed.

It is to be noted that the position of the high specific activity (Fig. 6) is approximately (*c.* 43 S) that in which McCarthy *et al.*[15] observed a ribosomal precursor which they called neosomal. The high specific activity in this region is thus either a stable messenger or a comparatively stable ribosomal precursor. It is difficult at present to distinguish between these possibilities.

Effect of histones on RNA synthesis

The RNA polymerase of *B. megaterium* is present entirely in the nuclear fraction and none can be detected in the cytoplasmic. It is firmly bound and it has not been found possible to release it except by disrupting the DNA with DNAase[2]. The RNA polymerase can then be concentrated by the method used by Chamberlin and Berg[16].

B. megaterium like other bacterial cells does not appear to contain any of the type of basic protein called histones[1]. It was suggested a long time ago by Stedman and Stedman[17] that histones act by suppressing the gene function of the DNA they are combined with. The availability of the polymerase which synthesises RNA on the DNA template has made it possible to test this idea. Huang and Bonner[18] found that using native nucleohistone prepared from pea seedlings and a polymerase from the same source, little RNA synthesis occurred, although the DNA by itself was active. In reconstituted nucleohistone made by adding various fractions of histone to DNA in strong solutions and then dialysing away the salt, various degrees of inhibition have been observed[19,20].

Our own observations which have been partially reported[21] come under two headings:

(1) the effect of calf thymus histone on RNA synthesis by the isolated nuclear material of *B. megaterium* without any added polymerase.

(2) the effect of calf thymus histone and histone fractions on RNA synthesis, using calf thymus histone and histone fractions with calf thymus DNA as a template.

In these experiments it has been found difficult to prepare "soluble" nucleoproteins as it was found that after dialysing the mixture of DNA and histone, gelatinous materials were obtained with which experiments were usually performed.

The following results were obtained: (i) Calf thymus histone has only a small effect on the rate of synthesis of RNA by the nuclear material of *B. megaterium* (Table 2). It can be seen that the effect of an excess of histone is not more than 25%. This may be due to the fact that the DNA of the nuclear material is already combined with protein; although not of a basic character, which prevents the added histone combining with the DNA. (ii) Calf thymus histone decreases the synthesis of RNA using calf thymus DNA as a primer (Fig. 7). The inhibition is much less than in a natural nucleohistone which corresponds approximately with the final point in composition. (iii) All the various fractions of calf thymus histone which have been described[22], have a marked inhibitory effect on DNA synthesis and there is no great difference

TABLE 2—INCORPORATION OF ^{3}H-URIDINE TRIPHOSPHATE INTO THE NUCLEAR FRACTION OF *B. megaterium*

	Control	100% Histone	200% Histone	Boiled control
C.p.m./mg. RNA	12,300	11,200	8,700	75

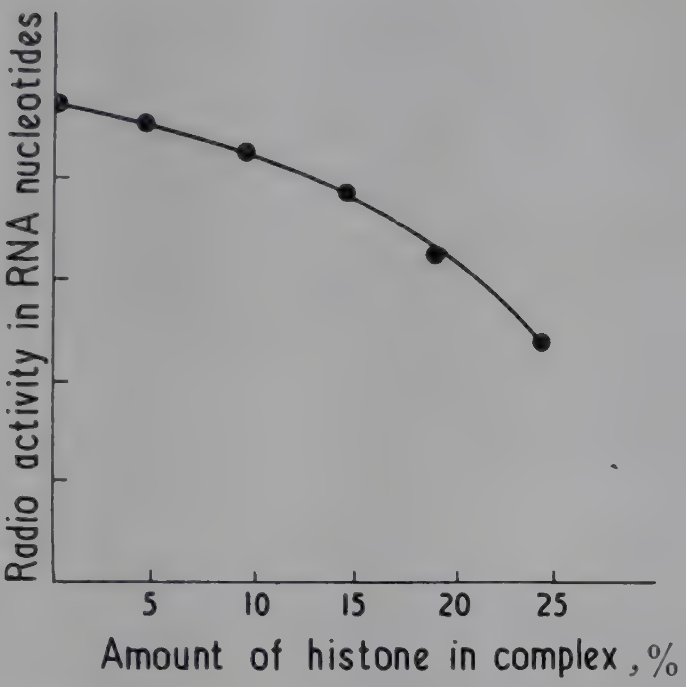

FIG. 7—EFFECT OF WHOLE HISTONE FROM CALF THYMUS ON RNA SYNTHESIS WITH CALF THYMUS DNA AS PRIMER: Each assay contained 19 μg. of DNA with the amount shown of the whole histone

between the fractions, except that Fl (a very lysine-rich histone) appears to be somewhat more effective than the fractions F2a, F2b and F3[21] (Fig. 8).

The effects of histones do not appear to be very specific. Thus the histone F1 causes almost complete inhibition when present in the complex in the weight ratio 1 histone : 1 DNA, which is much greater than its ratio in the natural thymus nucleoproteins. This means that F1 inhibits much more than those parts of the DNA with which it was originally combined. It was also found that the synthetic polymer, polylysine caused almost total inhibition of RNA synthesis when present at a ratio of more than 1:1.

It is in fact doubtful if true reconstitution of a nucleoprotein can be achieved by mixing solutions of DNA and histones, followed by dialysis. When a solution of histone and DNA in 2*M* sodium chloride is dialysed, a precipitate is formed when the salt concentration reaches about 0·6 *N*. In this precipitate the DNA fibres are probably united by histone in a kind of network. On dialysing further the nucleoprotein may appear to go into solution and it is probable that under these circumstances a certain amount of alignment of the histone strands on the DNA fibres takes place.

It will be necessary to make further studies of this question in order to determine the functions of the individual histone fractions. The whole histone can be fractionated into 4 groups of compounds[17], each of which when studied with starch gel electrophoresis, is found to be complex. However, there does not seem to be a very large number of distinct species, which would be the case if each gene had a special histone. For example, it is found that the number of peptides formed from some fractions which have been

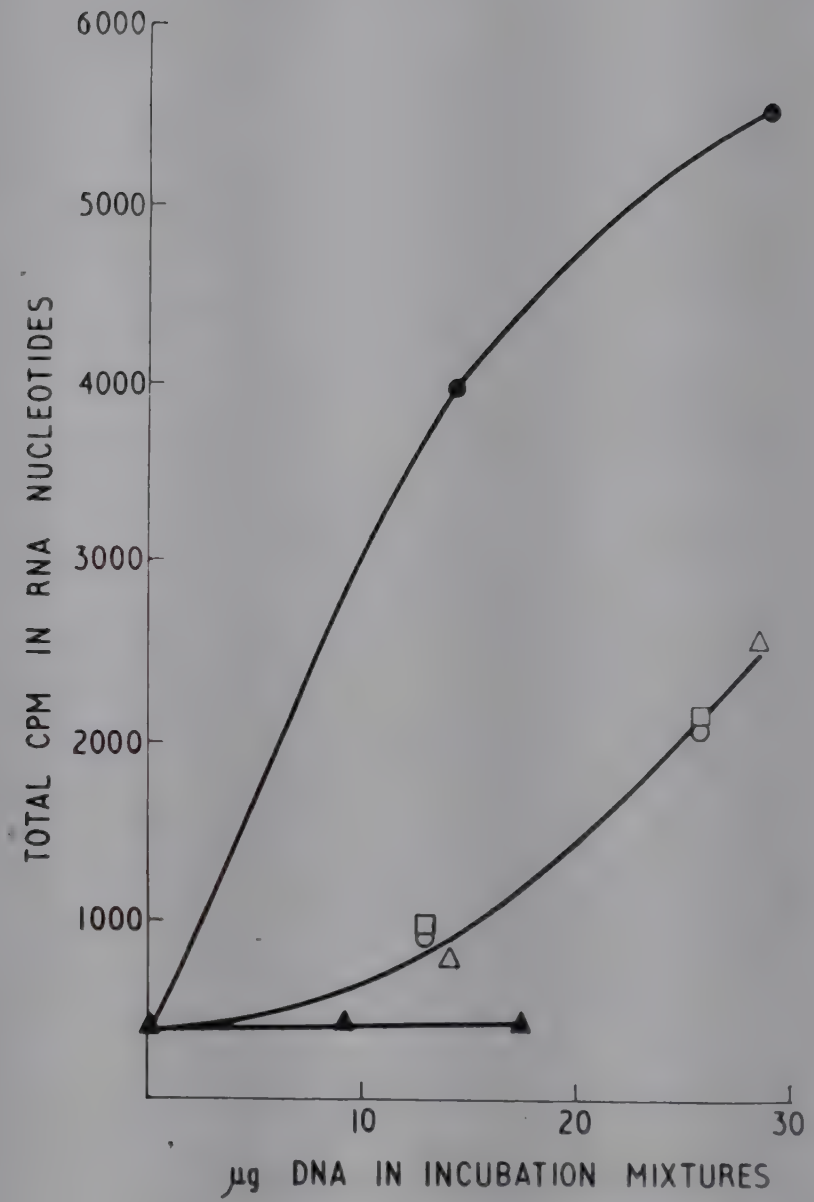

FIG. 8—EFFECT OF VARIOUS FRACTIONS OF CALF THYMUS HISTONE ON RNA SYNTHESIS BY CALF THYMUS DNA: From Barr and Butler[21]. The ratio DNA/histone is 1:1 (wt/wt). ● DNA alone, △ Fl-DNA complex, □ F2a-DNA complex, ○ F2b-DNA complex, ▲ F3-DNA complex

examined[23] (also, Phillips, D.M.P., unpublished data) is rather limited, and this does not suggest a very large number of distinct compounds within each fraction. Moreover, histones examined within mammalian species show a remarkable lack of species or tissue specificity[24]. It is suggested[21] therefore, that the histones do not distinguish individual genes, but classes of genes having different functions, e.g. operator genes and regulator and structural genes. It could well be that histones are involved in a "repressor system" similar to that postulated by Jacob and Monod[25], for bacteria but the manner in which particular types of histone could be added or removed from a gene or group of genes, has not become apparent. We would like to think that RNA is implicated in this process, but our experiments on this question are at a preliminary stage.

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Induction and Repression of Enzymes in *Lactobacillus plantarum*

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It is well known that the induction and repression of enzymes play an important role in the regulation of metabolism at the genetic level. Considerable knowledge has been acquired regarding the mechanism of induction and repression of enzymes by the exhaustive studies of Jacob, Monod, Cohn, Pardee and others. Most of their work has, however, been confined to the induction of the enzyme β-galactosidase in *Escherichia coli*. According to the widely accepted hypothesis of Jacob and Monod[1] the repressor molecules produced by the regulatory genes block the synthesis of proteins by the structural genes through the interference with the operator gene. In this Symposium Dr Novick has presented evidence elucidating the nature of the repressors which are most probably protein molecules. My interest, however, is limited in the sense that I thought of studying induction and repression of enzymes in *Lactobacillus plantarum*. Studies of different systems might eventually help towards the elucidation of the mechanism of induction and repression of enzymes. *L. plantarum* may be adapted on various sugars including pentoses. Horecker and his group[2] have worked out the pathway of metabolism of a number of pentoses by this microorganism. I thought of concentrating on the pathway of metabolism of one pentose, i.e. L-arabinose, and in the beginning to confine the studies only to the first enzyme of the series, i.e. L-arabinose isomerase.

The pathway of the metabolism of L-arabinose as worked out by Burma and Horecker[3] is shown in Fig. 1.

Lee and Englesberg[4] have mapped the positions of these enzymes on the genome of *Esch. coli*. They are carrying out extensive studies on the induction and the regulation of these enzymes at the genetic level. I will mostly deal with the induction and repression of the first enzyme, L-arabinose

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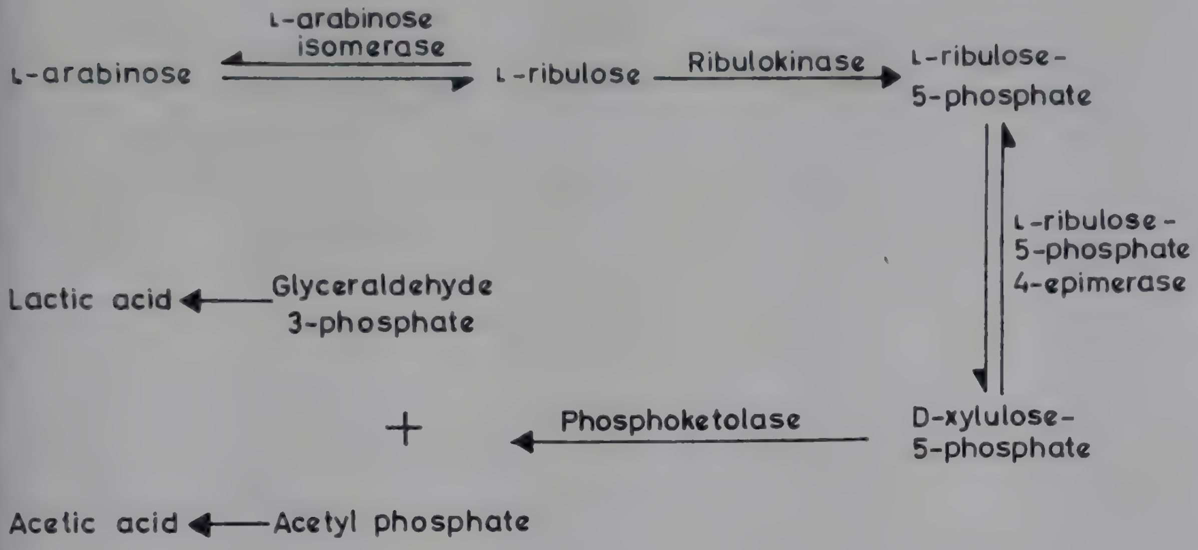
L-arabinose isomerase
L-arabinose
L-ribulose
Ribulokinase
L-ribulose-5-phosphate
L-ribulose-5-phosphate 4-epimerase
Lactic acid
Glyceraldehyde 3-phosphate
+
Phosphoketolase
D-xylulose-5-phosphate
Acetic acid
Acetyl phosphate

FIG. 1—PATHWAY OF L-ARABINOSE METABOLISM BY *L. plantarum*

isomerase, in *L. plantarum*. Some of the results will appear in a forthcoming publication[5].

Before discussing the experiments dealing with the induction of L-arabinose isomerase, let us have a look at the growth of *L. plantarum* on arabinose. Figs. 2 and 3 elucidate the nature of growth of the microorganism on arabinose before and after adaptation. The glucose-grown cells have a long lag period for growth on arabinose which is not evident in case of arabinose-adapted cells. If the arabinose-adapted cells are inoculated into glucose medium and grown for another 12 hr, they do not show the lag period as in the case of glucose-grown cells (Fig. 2). However, if the cells are transferred consecutively three times to the glucose containing medium and each time the cells are allowed to grow for 10-12 hr, and finally they are grown on arabinose, the cells behave like the glucose-grown cells as if they had no experience with arabinose (Fig. 3). The same situation was found to be true for growth on xylose. This represents a case of enzyme induction and repression. To study the induction of L-arabinose isomerase the enzymatic reaction was followed by the cysteine carbazole colour reaction of the pentulose formed in the reaction[6]. The cells were toluenized[7] and the toluenized cell suspension was used in the assay system. The rate of reaction was proportional to the enzyme content within limited range[5]. All the assays were done within that range. The amount of enzyme activity as measured in toluenized cells was equal to the total amount of enzyme present within the cell[5].

For inducing the enzyme L-arabinose isomerase the glucose-grown cells were washed, suspended in fresh medium containing the inducer and incubated at 35°C. After requisite period, the cells were treated with toluene

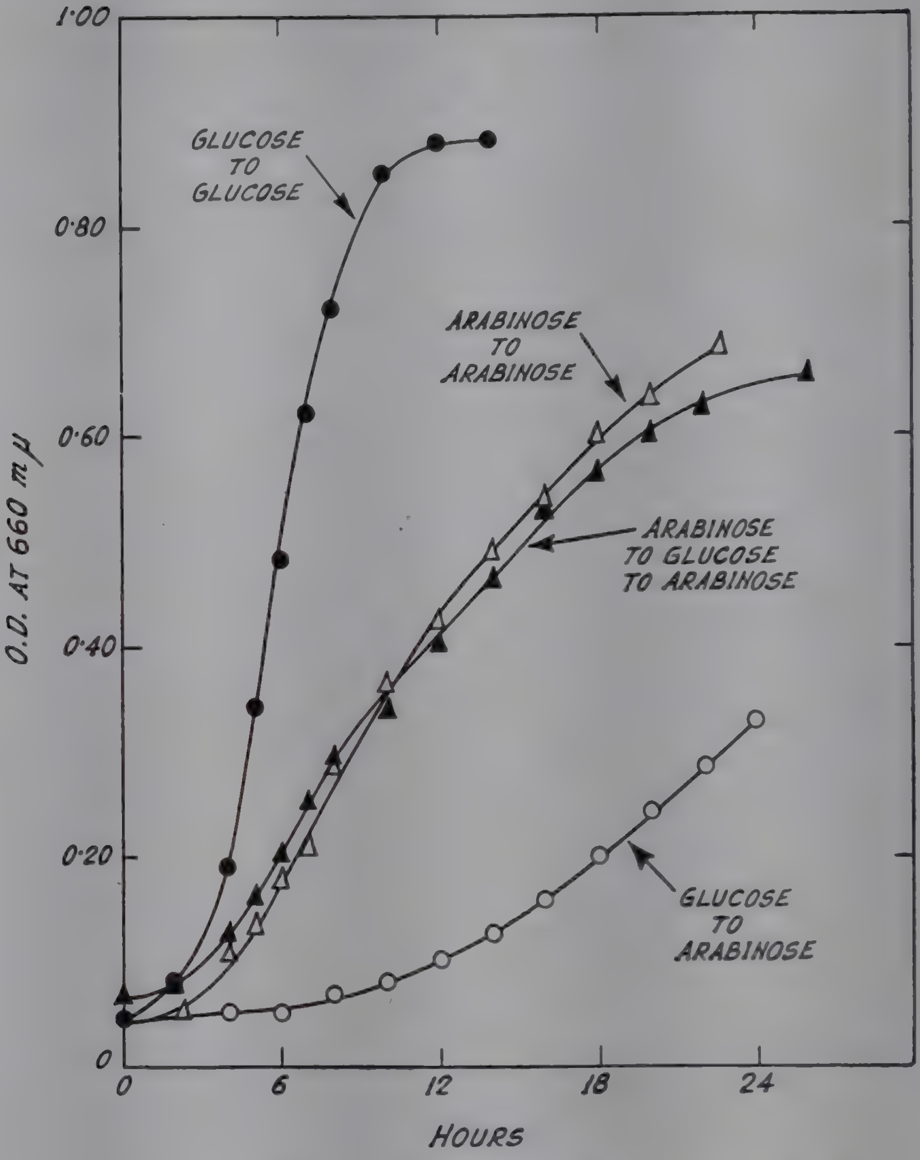

FIG. 2—GROWTH OF *L. plantarum* ON GLUCOSE AND ARABINOSE

for 30 min. The toluenized cells were collected by centrifugation and suspended in Tris (0·05 *M*; *p*H 7·3). The kinetics of induction of L-arabinose isomerase is shown in Fig. 4. After a lag period of about 30 min., the synthesis of the enzyme continues at a linear rate and reaches the maximum level within 3-4 hr. During this period there was very little growth as is evident from the growth curve presented in the same figure. Attempt was made without success to induce the enzyme in cells suspended in buffer or in basal medium as described by Wright and Skeggs[8]. The enzyme can be induced in cells suspended only in enriched medium as described by Stumpf and Horecker[9]. It was thought that some factors, as in the case of glycerol oxidation[10], may be required for the induction process. Hence, the minimal medium was supplemented with either tryptone (10 μg./ml.) or yeast extract (10 μg./ml.), or a mixture of the two, or glucose (1 μg./ml.), but in no case enzyme induction was observed.

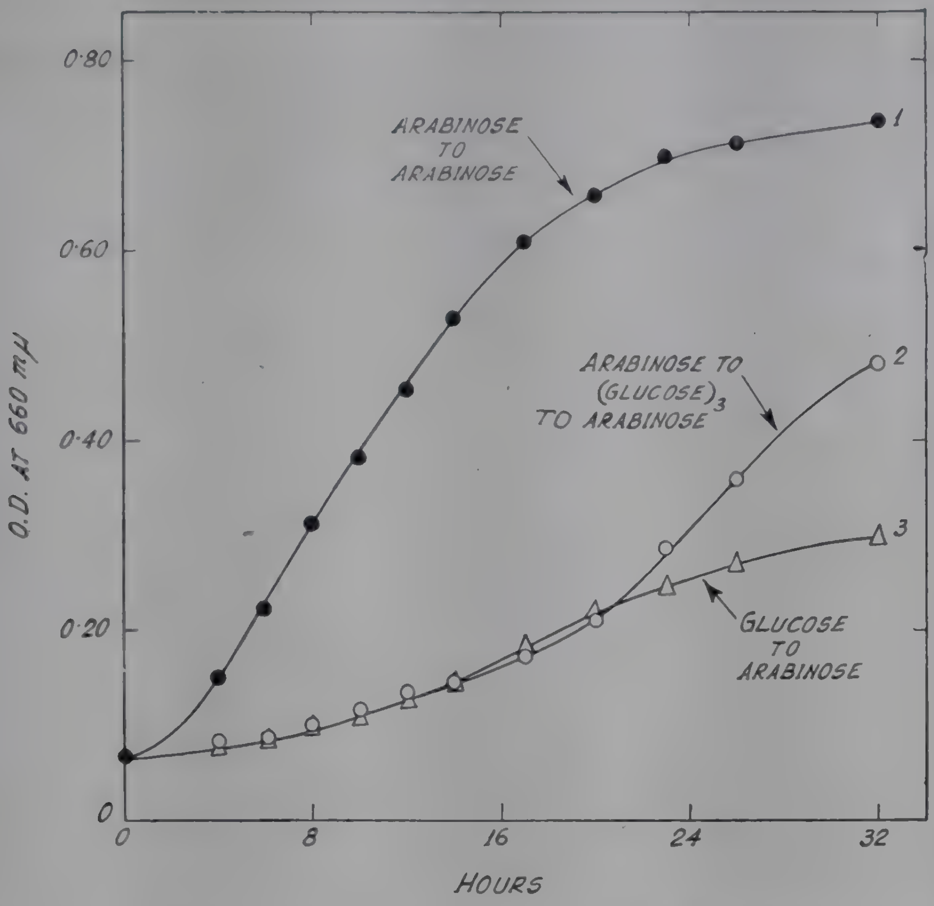

FIG. 3—GROWTH OF *L. plantarum* ON ARABINOSE UNDER DIFFERENT CONDITIONS OF ADAPTATION

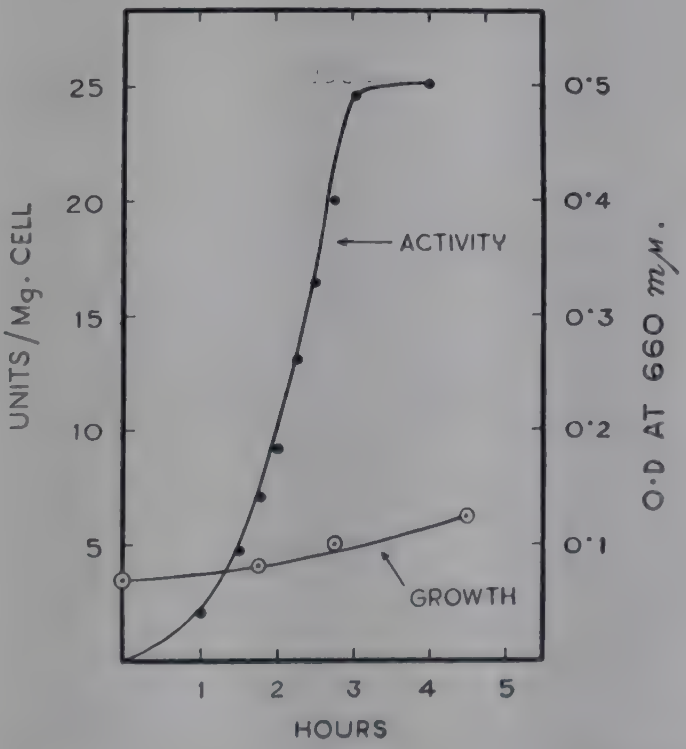

FIG. 4—INDUCTION OF L-ARABINOSE ISOMERASE IN *L. plantarum*

Though D-xylose isomerase, isolated from the extracts of D-xylose grown cells, was reported by Burma and Horecker[3] to have L-arabinose isomerase activity, no L-arabinose isomerase activity could be induced with D-xylose under the conditions described above. Excepting L-arabinose which is the substrate of the enzyme, other sugars like D-arabinose, D-xylose, D-ribose, etc. failed to act as inducer. No substrate analogue was available for testing as inducer. The concentration of L-arabinose, ranging from 0·05 to 2·0%, has no effect on either the rate of enzyme formation or the total amount of enzyme produced. In all experiments 0.1 % of L-arabinose was used unless otherwise stated.

While performing these induction experiments it was observed that if instead of washing the glucose-grown cells and then suspending them in fresh medium containing the inducer, the glucose-grown cells were directly diluted with fresh medium containing the inducer, the lag period required for induction was prolonged. Naturally, it was suspected that some inhibitor was present in the glucose-grown inoculum. Glucose was the first compound to be suspected as it is a well known repressor of enzyme induction.

The repressor action of glucose on the induction of L-arabinose isomerase is shown in Fig. 5. In this experiment two concentrations of glucose were used, 0·05 and 0·20%. The results indicate that the addition of glucose immediately stops any further synthesis of the enzyme as the total amount

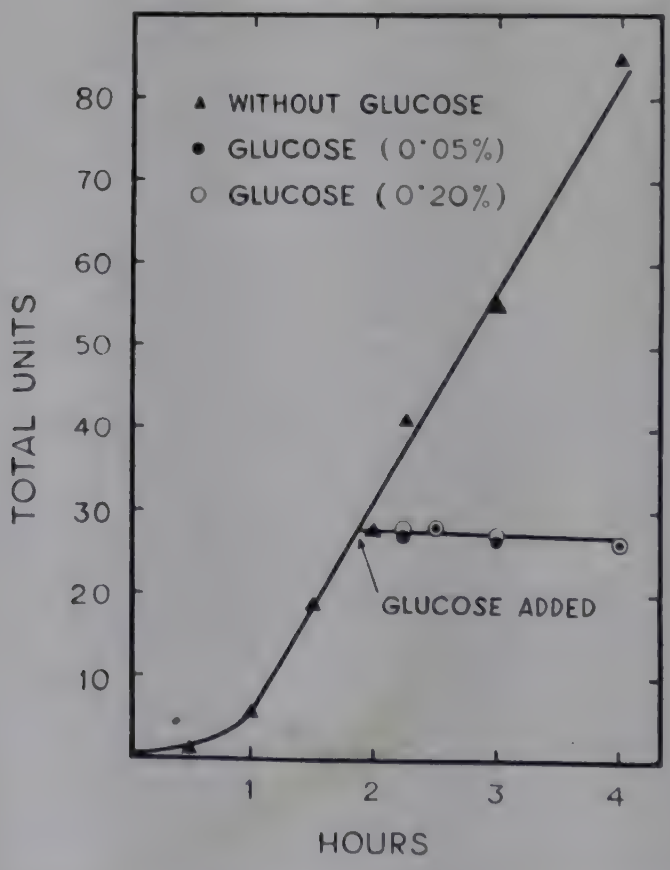

FIG. 5—EFFECT OF GLUCOSE ON THE INDUCTION OF L-ARABINOSE ISOMERASE

of enzyme in the cell suspension remains constant after the addition of glucose, whereas the total amount of enzyme in the control cells increases linearly with time. This inhibitory effect of glucose is definitely not due to feedback inhibition as in that case one should have expected decrease in the enzyme activity instead of maintenance of the constant level of the enzyme. The same argument holds good for the possibility that the glucose effect is not due to inefficient toluenization in presence of glucose which may be true for many other systems. Moreover, it was previously checked that the presence of glucose during toluenization did not interfere with the assay of L-arabinose isomerase in *L. plantarum*.

This type of catabolite repression was also discernible with D-fructose, D-mannose and mannitol. D-Xylose, D-glycerol and glucuronate, however, have no effect on the induction of the enzyme[5]. The extent of repression depends on the relative concentrations of the inducer and the repressor. The catabolite repression has been studied by a number of workers including Magsanik[11]. The mechanism of catabolite repression, however, is not very clear. It was observed that sugars which are better metabolites and thus lead to better growth of the cells have more repressive action on the induction of enzymes involved in the metabolism of carbohydrates. This is also found to be true for the enzyme L-arabinose isomerase.

The next question was why such a long lag period was required before the induction of the enzyme could start? Is this lag period any way connected with the lag period required for growth ? Or, is this lag period required for entry of the substrate within the cell and to build up the requisite pool for induction? To get an answer to this question the enzyme was induced in cells which were growing exponentially on xylose. Xylose is known not to repress the induction of the enzyme. Even in that case there was a delay of about 30 min. before the synthesis of the enzyme could start and the kinetics of induction followed the same course as in the case of glucose-grown cells. Indirect evidence favours the possibility that the lag period of induction is required for the entry of the inducer within the cell and the building-up of suitable pool size. In Fig. 6, the kinetics of uptake of C^{14}-arabinose, the inducer, by glucose-grown and arabinose-adapted cells are shown. The experimental conditions were exactly the same as described for the induction experiments. Aliquots of the cell suspension were collected, chilled, washed, plated and then counted. In the case of arabinose-adapted cells, L-arabinose enters the cell without any lag period whereas in the case of glucose-grown cells the entry of L-arabinose starts after a lag period of 30-40 min. Still one has to be cautious in drawing any definite conclusion from these incorporation studies as the inducer itself is metabolized by the cell.

It is well established now that the information for the synthesis of the inducible enzymes is already present in the genetic material, DNA. Under certain conditions this message is transcribed in the form of messenger RNA. The synthesis of the messenger RNA is a prerequisite for any protein synthesis

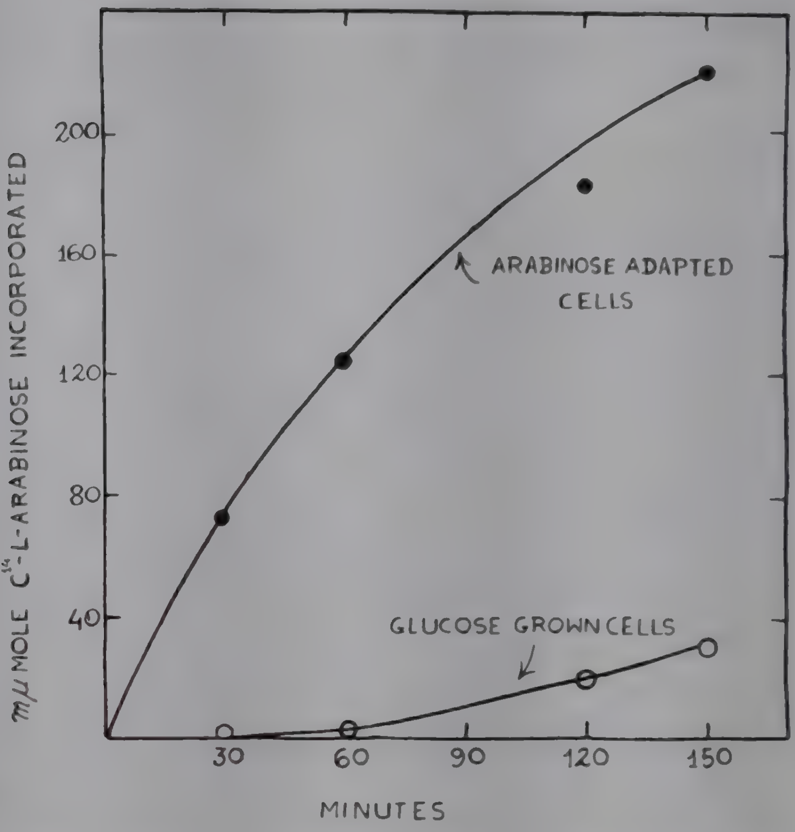

FIG. 6—KINETICS OF UPTAKE OF ^{14}C-ARABINOSE BY GLUCOSE-GROWN AND ARABINOSE-ADAPTED CELLS

including the synthesis of the inducible enzymes. So the effects of some antibiotics and mutagens which specifically interfere either with DNA or with RNA synthesis were studied to gain some knowledge about the mode of induction of L-arabinose isomerase. Actinomycin D, fluorouracil (FU) and mitomycin C were the compounds tested. Before investigating the effects of these compounds on the enzyme induction, their effects on the growth of *L. plantarum* were studied[5]. The growth study was also required to get an idea about the concentrations of these antibiotics or mutagens to be used in the induction experiments. In all the cases, there was no difference in the growth rates between the control and the treated cells up to 135 min. after the addition of the antibiotic or mutagen. Actinomycin D, first isolated by Waksman and his group, was found to inhibit the synthesis of cellular RNA but not of DNA or protein in mammalian[12] as well as in bacterial systems[13]. Though there was no difference in the growth rates between the control and the actinomycin D treated cells during the period over which the enzyme induction was followed, actinomycin D at a concentration of 0·1 μg./ml. did not allow the enzyme to be synthesized (Fig. 7). A period of 15-17 min. elapses before actinomycin D exerts its effect. This may be due to the fact that the messenger RNA already synthesized before the entry of actinomycin D, carries out the function for 15 min. Or, it could be that actinomycin D takes some time to enter within the cell. In this connection the work done by Pollock in *B. cereus* and *B. subtilis* may be

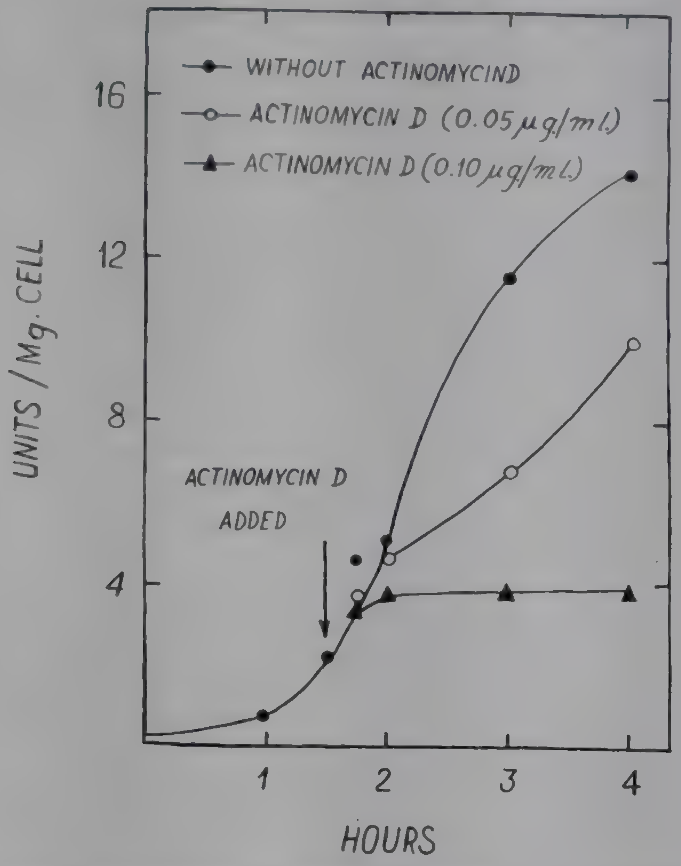

FIG. 7—EFFECT OF ACTINOMYCIN D ON THE INDUCTION OF L-ARABINOSE ISOMERASE

mentioned[14]. During induction of the enzyme penicillinase in inducible strains, enzyme synthesis continues uninhibited up to 40 min. after the addition of actinomycin D, whereas in case of the constitutive mutants the synthesis continues uninterrupted up to 4 hr. It has been suggested that the differential effect is due to the differences in the stability of messenger RNA under different conditions.

Fluorouracil has been one of the most useful analogues introduced by Heidelberger and his coworkers[15]. That fluorouracil is incorporated specifically into RNA in place of uracil has been well documented by Horowitz and Chargaff[16] as well as Champe and Benzer[17]. Under the conditions of enzyme induction, the messenger RNA for the enzyme induced is expected to be synthesized at the most rapid rate. Hence, the presence of FU during the period of induction may lead to the replacement of the uracil of the messenger RNA for the enzyme by fluorouracil. Unlike the effect of actinomycin D, that of FU on the induction of the enzyme L-arabinose isomerase (Fig. 8) reminds us of the effect of the antimetabolite on the growth rate. Actinomycin D combines with the guanine residues of DNA forming complexes so that the priming ability of the DNA is lost[18]. It is all or none phenomenon. On the other hand, the presence of FU does not inhibit the rate of messenger RNA synthesis though uracil is replaced by FU. FU of the messenger RNA may or may not make mistake while pairing with the adaptor RNA. It is a question of probability. Moreover, all the

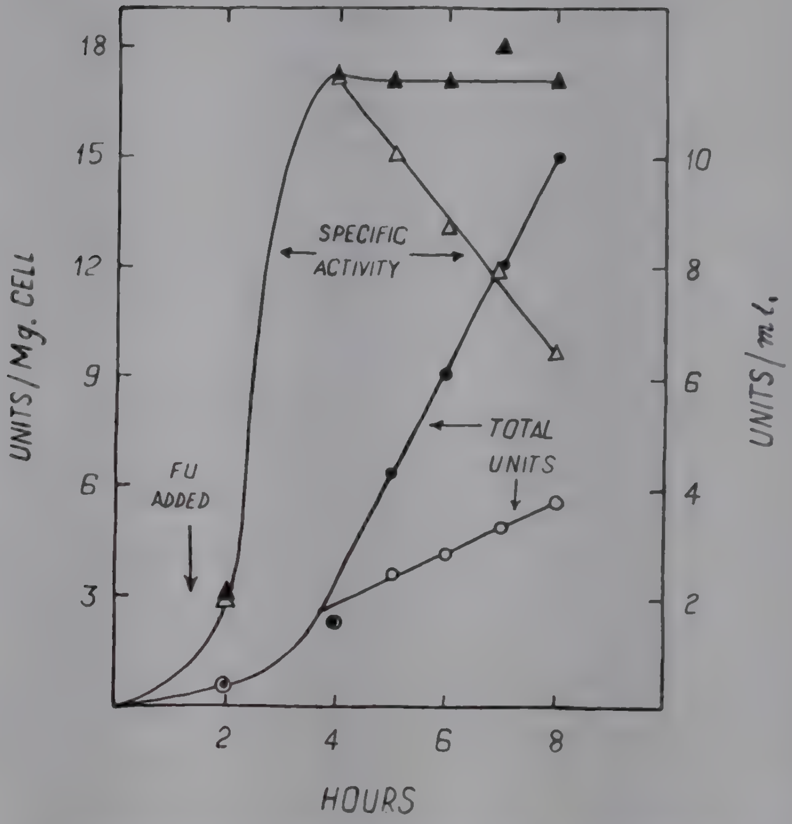

FIG. 8—EFFECT OF FLUOROURACIL ON THE INDUCTION OF L-ARABINOSE ISOMERASE: Without fluorouracil (▲, ●); with fluorouracil (△, ○)

uracil containing codes may not necessarily be involved in the attachment of the amino acids required for the enzymatic function of L-arabinose isomerase. Hence, prolonged incubation is required to have some detrimental effect on the synthesis of enzymatically active L-arabinose isomerase molecules. This situation is reflected in the experimental results presented in Fig. 8.

The antibiotic mitomycin C is known to interfere with DNA synthesis but not RNA and protein syntheses under *in vivo* conditions. Of course, there are a few instances where the enzyme induction is inhibited by mitomycin C[19]. As in the case of actinomycin D and FU the effect of mitomycin C on the growth of *L. plantarum* was followed (Fig. 9) before studying the effect of this antibiotic on the enzyme induction. The cells were treated with three different concentrations of mitomycin C (0·1, 0·5 and 1 μg./ml.). After incubation for 3-4 hr when the cells just start their logarithmic phase of growth, the inhibition of growth by mitomycin C became evident. There was marked inhibition of growth even at a concentration of 0·1 μg./ml. While carrying out the induction experiments, mitomycin C at a concentration of 0·1 μg./ml. was added after 60 min. As indicated in Fig. 10, the synthesis of the enzyme is not affected by the presence of mitomycin C. This result supports the evidence obtained by others working with microorganisms and mammalian systems[20,21]. Though mitomycin C affects irreversibly the duplication of DNA by binding with the DNA strands,

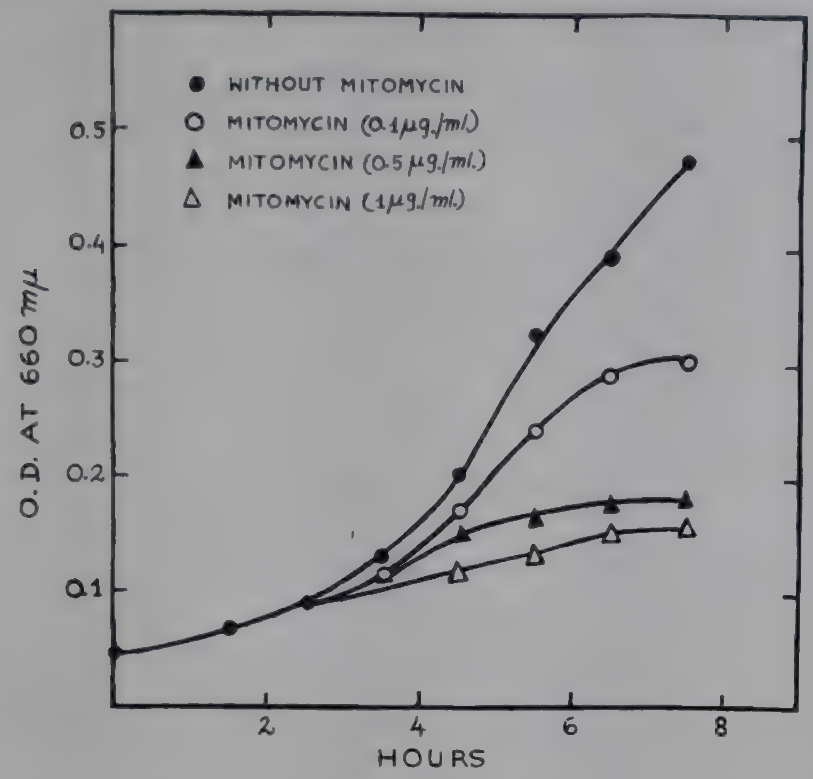

FIG. 9—EFFECT OF MITOMYCIN C ON THE GROWTH OF *L. plantarum*

DNA-mitomycin C complex can still act as a template for the synthesis of messenger RNA and thus does not affect RNA and protein syntheses within the cell.

So far, I was discussing the induction of the enzyme L-arabinose isomerase involved in the metabolism of a pentose, L-arabinose. Now I would like to digress a little by discussing the induction of another enzyme in the same microorganism and comparing the salient points in the induction of these two enzymes.

Lactobacillus plantarum can be grown on a number of hexoses as well. In order to grow on mannose this microorganism does not require any adaptation and the growth rate on mannose is almost the same as that on glucose (Fig. 11). On mannitol, however, a little longer lag period was required before the start of the growth. From the nature of growth on mannitol it appears that some enzymes involved in the metabolism of mannitol have to be induced. The enzyme mannitol dehydrogenase, which can oxidize mannitol to fructose, was looked for in the extracts of mannitol grown *L. plantarum*, but there was no indication of the presence of mannitol dehydrogenase. On the other hand, the enzyme mannitol-l-phosphate dehydrogenase which catalyses the conversion of mannitol-l-phosphate to fructose-6-phosphate was found to be present in considerable amounts. The level of the enzyme mannitol-l-phosphate dehydrogenase increases about 10-fold in the mannitol-grown cells in comparison with that in the glucose-grown cells (Table 1). This enzyme was found to be present in fructose-grown cells as well but the level was low as in the cells grown on

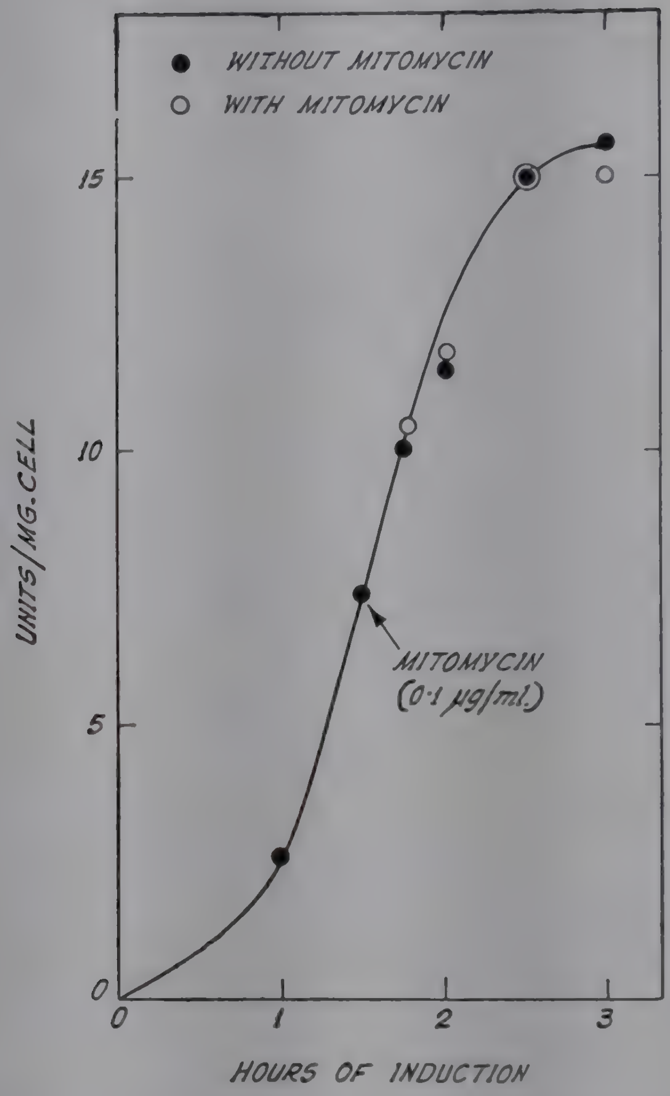

FIG. 10—EFFECT OF MITOMYCIN C ON THE INDUCTION OF L-ARABINOSE ISOMERASE

TABLE 1—MANNITOL-1-PHOSPHATE DEHYDROGENASE ACTIVITY IN EXTRACTS OF *L. plantarum* GROWN ON DIFFERENT SUGARS

Sugars on which cells were grown	Units/mg. protein
Mannitol	5·0
D-Glucose	0·5
D-Fructose	0·6
D-Xylose	0
L-Arabinose	0

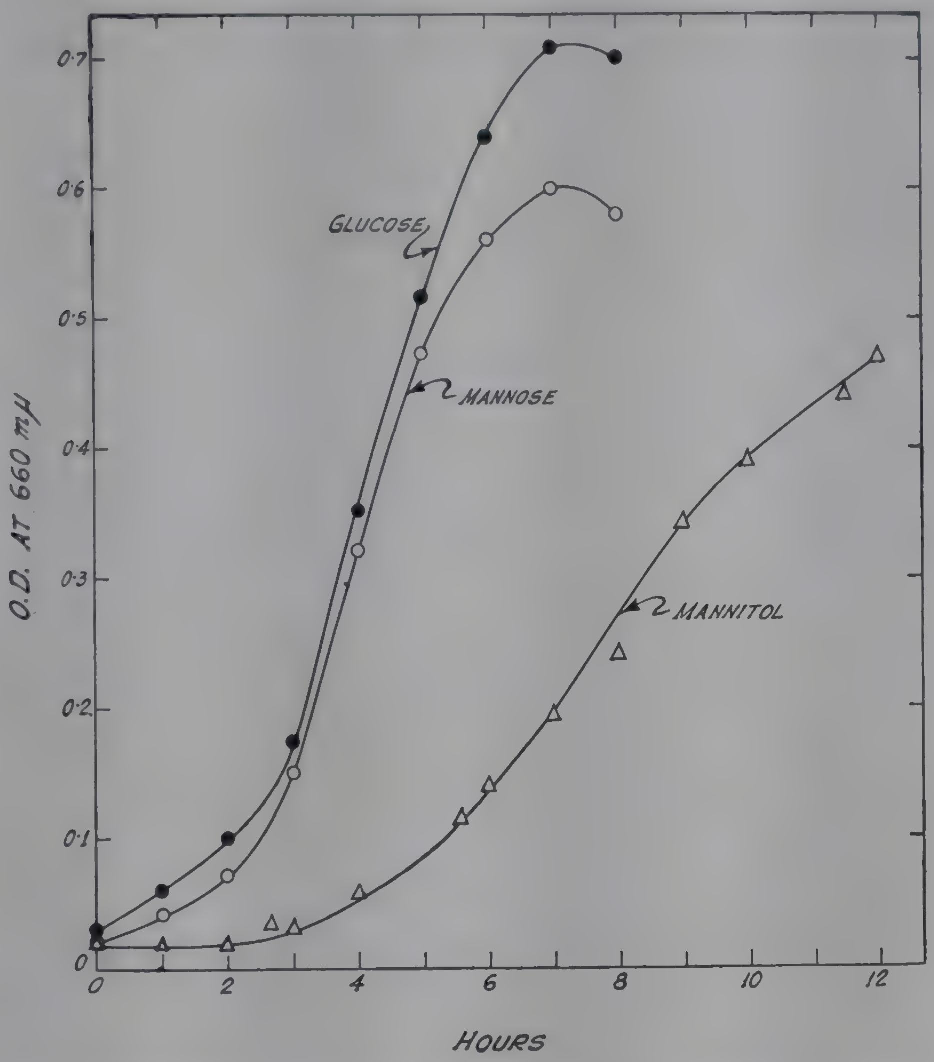

FIG. 11—GROWTH OF *L. plantarum* ON D-GLUCOSE, D-MANNOSE AND MANNITOL

glucose. The enzyme was, however, absent in arabinose or xylose-grown cells. Naturally, the enzyme was suspected to be involved in the metabolism of mannitol. The enzyme mannitol-l-phosphate dehydrogenase was first reported by Wolff and Kaplan[22] to be present in *Esch. coli* and was purified about 30-fold. The physiological function of the enzyme is not very clear as the kinase capable of converting mannitol to mannitol-l-phosphate has not so far been detected. The enzyme mannitol-l-phosphate dehydrogenase has been purified over 160-fold from the extracts of *L. plantarum*. The properties of the enzyme have also been studied in detail (unpublished results).

The enzyme mannitol-l-phosphate dehydrogenase can be induced in glucose-grown cells as well as in cells growing exponentially on arabinose (Fig. 12). The kinetics of induction are the same in both the cases as in the case of induction of L-arabinose isomerase it takes about 3 hr to attain the maximum level of the enzyme. While performing the induction experiments the enzyme was assayed in the toluenized cells by following DPNH oxidation at 340 mμ using fructose-6-phosphate as the substrate.

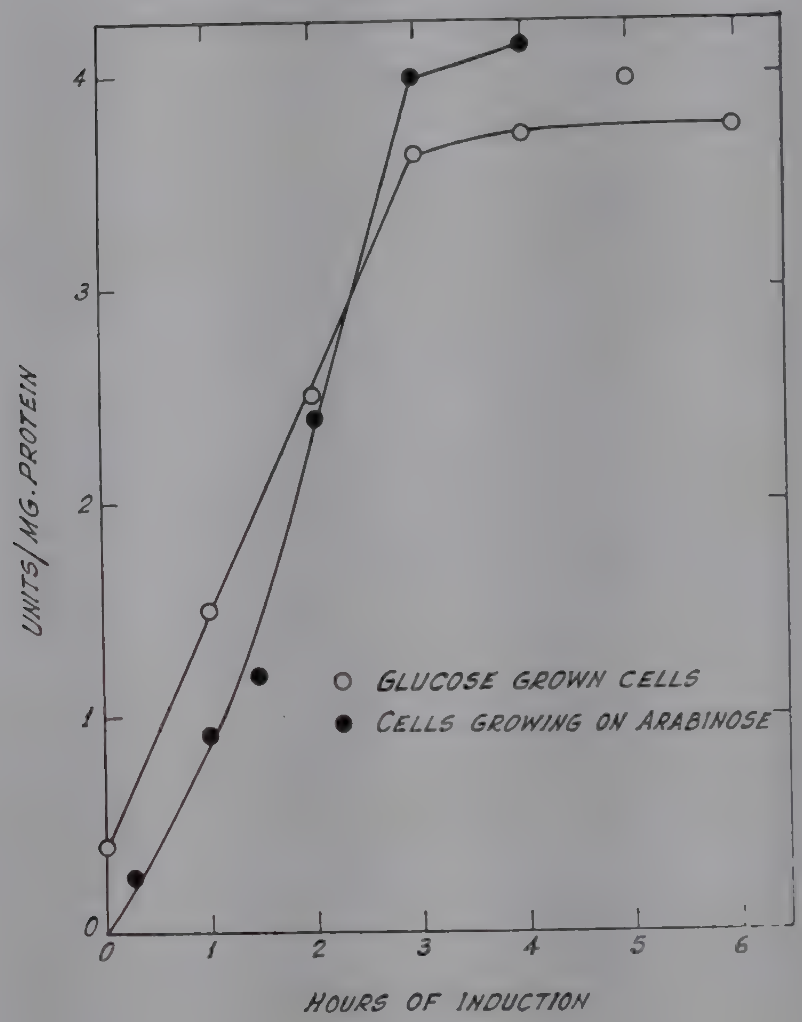

Fig. 12—Kinetics of induction of mannitol-1-phosphate dehydrogenase in glucose-grown and arabinose-adapted cells

The induction of mannitol-1-phosphate dehydrogenase as well is repressed by the presence of different catabolites (Table 2). There is greater repression by glucose and mannose whereas the repressor effects of fructose and arabinose are negligible. Xylose is almost without any affect. If we recall the nature of catabolite repression in the case of induction of L-arabinose isomerase, it will be obvious that the extent of repression was much more in the case of induction of L-arabinose isomerase. For L-arabinose isomerase, fructose is as good a repressor as glucose whereas it has little effect on the induction of mannitol-1-phosphate dehydrogenase.

The effect of actinomycin D on the induction of mannitol-1-phosphate dehydrogenase is presented in Fig. 13. Actinomycin D was used at a concentration of 0·1 μg./ml. The antibiotic was added 60 min. after the addition of the inducer when the synthesis of the enzyme had already started. As shown in Fig. 13, the synthesis of the enzyme continues at the same rate as that of the control up to 120 min. after the addition of actinomycin D, after which the synthesis completely ceases. The same concentration of actinomycin D (0·1 μg./ml.) stops the induction of L-arabinose isomerase within 15-17 min. (Fig. 7) after the addition of the antibiotic. If we accept

TABLE 2—EFFECT OF DIFFERENT CATABOLITES ON THE INDUCTION OF MANNITOL-1-PHOSPHATE DEHYDROGENASE

Catabolites	Repression %
Mannitol (0·2%)	..
Mannitol (0·2%)+glucose (0·2%)	28
Mannitol (0·2%)+glucose (0·5%)	60
Mannitol (0·2%)+arabinose (0·2%)	3
Mannitol (0·2%)+arabinose (0·5%)	18
Mannitol (0·2%)+mannose (0·5%)	68
Mannitol (0·2%)+fructose (0·5%)	20
Mannitol (0·2%)+xylose (0·5%)	6

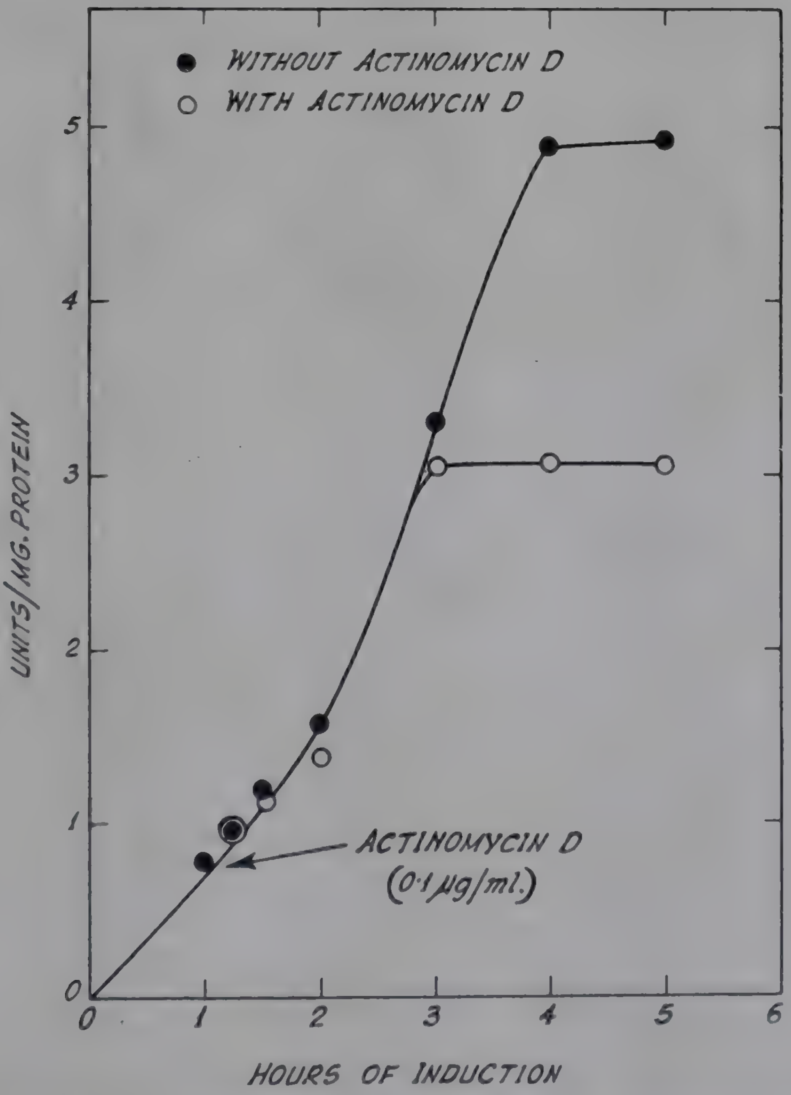

FIG. 13—EFFECT OF ACTINOMYCIN D ON THE INDUCTION OF MANNITOL-1-PHOSPHATE DEHYDROGENASE

the idea that these experiments with actinomycin D can tell about the stability of the messenger RNA, then we can assume that the messenger RNA for mannitol-1-phosphate dehydrogenase is much more stable than the messenger RNA of L-arabinose isomerase.

These are preliminary observations made in connection with the induction of two enzymes, L-arabinose isomerase and mannitol-1-phosphate dehydrogenase, in *L. plantarum.* The general processes of induction and catabolite repression observed are more or less similar to those observed in cases of other inducible enzymes. The use of the antibiotics and anti-metabolites like actinomycin D, fluorouracil and mitomycin C have elicited some preliminary information. Detailed investigations have to be carried out to throw light on the mechanism of induction. Isolation of mutants of *L. plantarum* and their use in the elucidation of the mechanism of induction and repression are aimed at.

ACKNOWLEDGEMENT

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The Genetic Code: Co-linearity and Interlacing

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It has been assumed for some time now that the gene and the polypeptide chain for which it codes are co-linear. That is, that the order of codons along the nucleic acid is the same as the order of the corresponding amino acids in the polypeptide chain. Until recently there was very little direct evidence for this, although it was known in a few cases that mutations close together in a gene affected the same or adjacent amino acids in the protein[1,2].

Two groups of workers have now presented evidence that is highly compatible with co-linearity. At the Cold Spring Harbour Symposium in June 1963, Dr C. Yanofsky gave the first data for the A protein of the tryptophane synthetase of *Escherichia coli.* He has shown, in several papers now in the process of publication[3–5], that over a length of about 60 amino acids, covering part of the polypeptide chain of this protein, the order found by genetic methods for 7 distinct sites is the same as the order of the corresponding amino acid alterations in the protein. Moreover, he has shown a rough correspondence between genetic map distance and distance apart on the polypeptide chain.

The other work comes from our laboratory at Cambridge in collaboration with Dr Bolle of the Institute de Biologie Moleculaire, Universite de Genève (Sarabhai *et al.*[6], and refers to the main protein of the head of bacteriophage T4D. They showed that a certain class of mutants, known as "amber" mutants, have the effect of terminating the polypeptide chain (when the host cell is *Esch. coli* B), so that instead of the complete chain the infected cell produces only a fragment of the chain, probably the stretch from the N-terminal end to the point corresponding to the amber mutation. They studied ten distinct mutants. By examining the trypsin or chymotrypsin digests of the infected cell they were able to order the different fragments according to their length. By this method they could order 8 segments of the chain, and the order thus found corresponded exactly to the order predicted from the genetic map constructed by using 2-point and

3-point crosses. For fuller details the reader is referred to the original paper.

It is not clear exactly what length of polypeptide chain this region covers, but a good guess would be, say, 300 or 600 amino acids. Thus their results are on a coarser scale than those found by Dr Yanofsky and his coworkers.

Both their results suggest that to a good first approximation the gene and the protein are co-linear. They do not establish that this co-linearity is metrical as well as being topological. By metrical I mean that the *distance* between two codons on the nucleic acid is proportioned to the *distance* between the two corresponding amino acids in the polypeptide chain. Both results suggest this is very roughly true. However, we have no direct measure of distance along the nucleic acid, but only the genetic map distance, and this depends on the process of genetic recombination. It is in fact highly unlikely that in phage the map distance is directly proportioned to the actual distance, because of the phenomenon of negative interference. There may, of course, turn out to be a single mapping fraction which will convert the map distance into the real distance, but it would not be surprising if the recombination frequency depended somewhat on the local base sequence, in which case no single mapping fraction would be possible. At the moment, in fact, it is plausible to assume that the nucleic acid distance and the polypeptide distance are indeed strictly proportional, and to regard the observed genetic map distances as telling us something about the process of recombination.

We now have fairly good though indirect evidence that the genetic code is non-overlapping (Wittmann, this Symposium), that the codons are triplets[7] and that a gene and its protein are co-linear. It might be thought that if we accept all this evidence the general nature of the genetic code could be considered established. However, it should be noted that we have no direct evidence that the three bases of a single codon are actually adjacent on the nucleic acid chain. All our present results could be explained by a code of the following type, which I have called an "interlacing" code: (1) the three bases of a codon are equally spaced, with $(n-1)$ bases between the first two (or the last two) bases of the codon (n is any integer greater than 1). (2) the reading mechanism moves on 3 bases on each step. Thus, for $n=2$, the first codon consists of bases 1, 3, 5; the second codon consists of bases 4, 6, 8; and the third codon consists of bases 7, 9, 11.

It is easy to see that n can be any integer except a multiple of 3, since in those cases it would generate an overlapping code, which is contradicted by the evidence. Medvedev[8] had previously suggested a code in which the three bases of a codon were not adjacent, but his code was an overlapping one.

Although such a code would obey the definition of co-linearity given in the second sentence of this paper, it should lead to non-linear anomalies when very fine genetic mapping is combined with amino acid replacement studies. Then in the example given above, fine genetic mapping would put bases 3, 4 and 5 in this order, but bases 3 and 5 would change the first amino acid, whereas base 4, which is in between them, would change the second amino acid. If one went along the nucleic acid chain altering each base in turn the alternations in the amino acids would be in succession 1, 1, 2, 1, 2, 3, 2, 3, 4, 3, etc. instead of 1, 1, 1, 2, 2, 2, 3, 3, 3, 4, etc. which we should obtain if the three bases of each codon were adjacent.

The fact that Yanofsky has not so far observed anomalies of this type makes it rather unlikely that the genetic code is interlacing. However, such a negative argument cannot be given much weight until more cases have accumulated, if only because some potential amino acid changes cannot be observed for technical reasons, e.g. they may have little or no effect on enzyme activity.

The best argument against interlacing codes is that, as far as I can see, there is no strong reason why the three bases of a codon should not be adjacent, nor any obvious advantage in having an interlacing code. An interlacing code (for n=2, or for higher numbers) is a theoretical possibility; it will have to be proved or disproved, but it is not very likely to exist.

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Studies on Infective DNA from Vaccinia Virus

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The isolation of infective RNA from tobacco mosaic virus by Gierer and Schramm[1] has been followed by reports at regular intervals of the isolation of infective nucleic acids from various viruses[2,3] and tumour agents[4–6]. The purpose of this paper is to report the isolation of infective DNA from Vaccinia virus and to provide evidence to suggest that this DNA may be single-stranded.

We had thought that this was the first report on the isolation of infective DNA from vaccinia, but Dr Zachau has informed us that Abel's studies[7] on the decoating enzyme of this virus have led on to the isolation of infective DNA.

That the nucleic acid of Vaccinia virus is essentially DNA has been known for some time[8,9]. Most pertinent to our studies are the investigations of Pfau and McCrea[10] in America, who showed that about 20% of the virus DNA could be isolated by a phenol-deoxycholate extraction procedure and that this DNA was single-stranded. They later isolated the virus DNA in nearly quantitative yields by the 2-mercaptoethanol-pronase pricedure[11]. This DNA was found by them to be largely double-stranded.

Our first experiments were carried out with unpurified virus to find out if the isolation of infective DNA from this virus was at all possible.

A strain of Vaccinia virus, obtained from Guindy (Madras), was maintained by serial passage on the chorioallantoic membrane (CAM) of 10 to 12-day old chick embryos. Suspensions of unpurified virus were made by grinding heavily infected CAMs in allantoic fluid or $0{\cdot}01M$ phosphate buffer of *p*H 7·2 containing 4% NaCl (1 ml./4 membranes), and centrifugation at 2000 r.p.m. Subjection of these virus suspensions to the phenol extraction procedure in the usual way followed by two successive ethanol precipitations yielded a mixture of nucleic acids.

The significant features about this nucleic acid mixture, which must have originated from the host as well as the virus were:

(1) It was essentially a mixture of RNA and DNA and was not contaminated with protein since its ultraviolet absorption at 260 and 280 mμ had a ratio of 2·0 and an HCl hydrolysate on paper chromatography gave no ninhydrin positive zones.
(2) It was infective as judged by its ability to infect CAM cultures and thereby forming pock-forming units in chick embryos that could be serially passaged (4 times).
(3) Infectivity of this nucleic acid mixture was lost on treatment with deoxyribonuclease.

It, therefore, seemed apparent that Vaccinia virus contained an isolable infective DNA. The isolation of infective DNA from purified virus was therefore undertaken.

For purpose of obtaining pure virus, virus suspensions in phosphate buffered saline (*p*H 7·2) containing 0·1% $MgCl_2 \cdot 6H_2O$ were treated with ribonuclease and deoxyribonuclease over 45 min. at room temperature (28°C.). The virus was then collected by centrifugation in a Spinco preparative model centrifuge using Rotor No. 40 at 40,000 r.p.m. for 3·5 hr. The virus pellet was thoroughly washed with cold buffer-saline, resuspended in buffer-saline and centrifuged again. The purified virus pellet so obtained was re-suspended in buffer-saline. Residual nuclease activity was destroyed by a brief treatment with trypsin, prior to extractions with water-saturated phenol.

Application of the unmodified phenol extraction procedure to purified Vaccinia virus suspensions yielded only infective solutions but the quantity of DNA was too small for precipitation or characterization. The introduction of sodium deoxycholate to the extent of 1% in the first phenol extraction yielded more substantial amounts of DNA.

This DNA did not appear to be contaminated with RNA or peptides (colour tests[12]). Its ultraviolet absorption spectrum had a maximum at 260·5 mμ and the ratio of absorbance at 260 and 280 mμ was 1·85. This DNA had been isolated in an identical manner to Pfau and McCrea's single-stranded DNA. Our data, which are presented later, also indicate that this DNA must largely be single-stranded.

It was, however, not infective (the pocks formed were few and not passageable). This was a cause for some concern until it was found that loss in infectivity could be prevented to a considerable extent by adding RNA (in this case ex-thymus from the British Drug House) to the virus suspension prior to extraction with phenol-deoxycholate. The amount of RNA added was five times the amount of DNA isolatable from the virus by this procedure. It was thought unlikely that the presence of this RNA would alter very much the nature of the DNA isolated and the assumption, that the chemical properties of our purified DNA and infective Vaccinia virus DNA were similar, seemed justified.

Some idea of the infectivity of the various DNA solutions can be gauged

TABLE 1—INFECTIVITY OF VIRUS SUSPENSIONS

Virus	Pock-forming units in chick embryos
Unpurified virus	10^{12}
Virus from infection of stationary CAM with mixture of nucleic acids from unpurified virus	10^5
Purified virus	10^{10}
Virus from infection of stationary CAM with DNA from purified virus	10^3

from the data presented in Table 1. None of the nucleic acid solutions obtained in these studies was able to infect chick embryos directly. Stationary CAM cultures were, therefore, first infected with infective nucleic acid-containing solutions and the resulting pock-forming activity examined in 10 to 12-day old chick embryos. If obtained, this pock-forming activity was serially passaged thrice.

Evidence of single-stranded DNA

Sinsheimer's studies[13] on the DNA from bacteriophage ϕX174 have established the criteria for determining whether a DNA is single- or double-stranded.

The optical density at 260 mμ of solutions of Vaccinia virus DNA in 0·15 *M* sodium chloride—0·015 *M* sodium citrate (*p*H 7) heated to various temperatures and rapidly chilled[14] resulted in an overall optical density increase of 9%. In a parallel experiment with double-stranded thymus DNA, the characteristic rapid increase in optical density was observed between 80° and 95°.

It is well known that formaldehyde reacts with the non-hydrogen bonded amino groups of polynucleotides and nucleotides[15,16]. The ready reaction and resulting change in optical density has been associated with single-stranded structures. It may be seen from Table 2 that heating to 90°C. for 10 min. and rapid cooling prior to formaldehyde treatment has no effect on the substantial optical density change (21%) observed on treating Vaccinia virus DNA with formaldehyde. On the other hand, treatment of thymus DNA with formaldehyde results in a very small change in optical density. Heat treatment (and, therefore, formation of single strands from this double-stranded material), followed by treatment with formaldehyde, results in a marked increase in optical density.

It is also well known that double-stranded DNA is more extensively hydrolysed by DNase than single-stranded DNA[13]. The hyperchromicity observed on DNase treatment of thymus and Vaccinia virus DNA at the end of 30 min. was 30·4% and 6·5% respectively.

TABLE 2—EFFECT OF FORMALDEHYDE ON THE OPTICAL DENSITY OF VACCINIA VIRUS AND THYMUS DNA

DNA*	Increase in O.D. at 260 mμ %
Vaccinia virus DNA	21.0
Vaccinia virus DNA heated at 90°C. (10 min.) and cooled rapidly to 0° before HCHO addition	20·0
Thymus DNA	5·0
Thymus DNA heated at 90°C. (10 min.) and cooled rapidly to 0°C. before HCHO addition	33·0

* Solutions in 0·15 *M* sodium chloride–0·015 *M* sodium citrate (*p*H 7) in the presence of HCHO (2%) maintained at 37°C. for 6 hr and room temperature for 6 hr.

Base ratio determinations[17] indicated that Vaccinia virus DNA has guanine, 29·1; adenine, 36·0; cytosine, 19·0 and thymine, 15·9%. There is no indication of base pairing as required in a double-stranded structure.

As mentioned earlier double-stranded DNA has been isolated from this virus. In our studies, a somewhat degraded sample of this firmly bound DNA has been obtained by sonication (10 kc./s. for 10 min.) of the first phenol-deoxycholate extract. This DNA gives the typical thermal denaturation profile of a double-stranded DNA.

SUMMARY

1. Application of the phenol extraction procedure to unpurified Vaccinia virus suspension yields infective nucleic acid mixtures. Infectivity is lost on DNAase treatment.

2. Application of the deoxycholate-phenol extraction procedure to purified Vaccinia virus gives essentially single-stranded DNA but with loss of infectivity.

3. This loss in infectivity is prevented to some extent if RNA is added to the virus suspensions before the deoxycholate-phenol extraction.

4. Vaccinia virus might, therefore, have a single-stranded infective DNA, but this cannot be said for certain until the homogeneity of this DNA is established, particularly since the infectivity is observable in chick embryos only after a passage through CAM cultures. Infectivity could, therefore, be due to a very small amount of highly infective double-stranded DNA which might be present in the isolated DNA.

5. The presence of more firmly held double-stranded DNA has been confirmed.

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Studies on the Exocellular Deoxyribonuclease I of *Penicillium chrysogenum*

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Enzymes hydrolysing deoxyribonucleic acid (DNA) are reported to occur in all animal and plant cells, bacteria, viruses, serum, snake venom and culture filtrates of bacteria[1]. Studies on the different properties of these enzymes have also been reported[1].

The deoxyribonucleases (DNase) obtained from different sources have different *p*H optima, but generally they fall into two groups, one with a *p*H optimum between 4·2 and 5·5, designated DNase II by Cunningham and Laskowski[2], and the other with a *p*H optimum in the vicinity of 7·5, called DNase I[3]. Most cells contain representatives of both types of enzymes, but the acid enzymes are more predominant in the tissues[2,4]. For optimal enzyme activity, the acid enzymes require low concentrations of electrolytes[5] while the alkaline enzymes are activated most effectively by Mg^{2+}, with few exceptions[4,6]. Changes in the intracellular concentrations of these enzymes are reported to be correlated with post-X-irradiation injury[7,10], with age of the tissues[4,8,11,12], or with phage infection of bacteria[13].

DNases have been employed in the study of structural characteristics of DNA[14]. The bovine pancreatic DNase I attacks DNA molecules at C-3′ to P linkages in order to yield 5′-phosphates. It also hydrolyses trinucleotides but not the dinucleotides. It preferentially attacks UPpPY sequence[15]. In contrast to this, the DNase II obtained from calf spleen or DNase I obtained from *Micrococcus pyogenes* hydrolyses DNA by attacking C-5′ to P linkage in order to yield 3′-phosphates[16,17]. It has been reported[18] that DNA possessing transforming activity rapidly looses its biological activity as a result of enzymic degradation of the DNA. Based on this information, it may be possible to show that the tissue nucleases are concerned in the defence mechanism against viral infection[19].

Although several reports have appeared on the properties of DNases obtained from various bacterial, plant or animal sources, to our knowledge there are no reports on the properties of exocellular or intracellular DNases obtained from fungi. The present report is concerned with the studies on the properties of exocellular DNase I obtained from the culture filtrates of *Penicillium chrysogenum* HA 10, a high penicillin yielding strain. A comparison has also been made of the properties of the above DNase I with those of DNAses I obtained from other sources.

MATERIALS AND METHODS

Strain and method of fermentation. *Penicillium chrysogenum* HA 10, a commercial, high penicillin yielding strain (a selection from Wisconsin strain) was used for the study. The organism was maintained on M_2-agar slants. The composition of the slants medium was as follows: glycerine, 10 g.; yeast extract, 5 g.; glucose, 10 g.; NaCl, 10 g.; KH_2PO_4, 0·1 g.; $MgSO_4.7H_2O$, 0·05 g.; agar, 20 g.; the various constituents were dissolved and made to one litre with distilled water. The *p*H of the medium was adjusted to 7·0 before sterilization. The organism was grown by a two-step fermentation in shake flasks by submerged culture process using seed and the fermentation media of Jarvis and Johnson[20]. All the fermentations were carried out in duplicates using 500 ml. Erlenmeyer flasks containing 100 ml. medium. They were incubated at 24°C. on a rotary shaker making 250 r.p.m. and describing a circle of 2 in. in diameter. The seed medium (100 ml.) was inoculated with approximately 20 million spores, and after 2 days of incubation a 10 ml. aliquot of the vegetative growth was transferred into 100 ml. fermentation medium. The fermentation cycle was continued up to 5 days. Phenylacetic acid (0·1%) was added to the fermentation medium as a side chain precursor for production of penicillin G. Two flasks were removed every day and were processed in the following manner. The contents of the flasks were filtered under suction over a Buchner funnel, the culture filtrate was adjusted to *p*H 7·0, and refiltered over a fine filter paper (Whatman No. 42) without applying suction. One ml. of toluene was added as a preservative to the culture filtrate, which was stored in frozen condition. Penicillin in the culture filtrate was assayed by a modified iodometric method[21].

Measurement of enzyme activity. The procedure for the determination of DNase I activity was essentially the same as described before[22]. The test system consisted of a suitable aliquot of the enzyme (about 0·5 to 1·0 ml. of the culture filtrate), 1·0 ml. of Tris-(hydroxymethyl) aminomethane buffer (0·1 *M*; *p*H 7·3), 0·015 *M* $MgSO_4.7H_2O$, 2 mg. DNA (sodium salt, calf thymus, Hammersten highly polymerized; Mann Research Laboratory Inc., New York, N.Y.), and distilled water to make the total volume to 3·7 ml. Substrate and enzyme blanks were run simultaneously. The tubes were incubated at 45°C. for 2 hr. At the end of the incubation period, 0·8 ml. of trichloracetic acid (55%) was added to each tube, mixed thoroughly and allowed to stand for 10 min. in a chilled water-bath. The contents of the tube were then centrifuged and the clear supernatant was used for the determination of deoxyribose-purine-containing fragments by the colorimetric procedure of Allfrey and Mirsky[11]. Deoxyribose was used as a standard. A suitable aliquot of the trichloracetic acid supernatant was made to 1·5 ml. with distilled water and 3·0 ml. of diphenylamine reagent was added. The tubes were kept in boiling water-bath for 20 min., cooled and the colour density measured at 600 $m\mu$ in a Beckman spectrophotometer (model DU).

Comparison of the properties of the enzyme. The influence of *p*H and temperature on the stability of the human plasma DNase I of Cohn fraction VI-2, bovine pancreatic DNase I (Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.), and exocellular DNase I of *P. chrysogenum*, was studied. Fractionation of the plasma was carried out according to the procedure reported by Cohn *et al.*[23] and outlined by Keller and Block[24]. The solutions of bovine pancreatic DNase I and plasma DNase I in different buffers were prepared as described earlier[22]. For the determination of exocellular DNase I of *P. chrysogenum*, 1 ml. each of culture filtrate was added to 1 ml. of Tris buffer (0·1 *M*; *p*H 7·3). The final *p*H of the system was adjusted to 2·8, 7·0 and 9·0, respectively, by addition of HCl or NaOH. All the tubes were covered and placed in a boiling water-bath for 30 min., cooled and the *p*H of the solutions was adjusted to 7·0. DNase I activity was determined on suitable aliquots from these tubes as described above.

The effect of addition of magnesium and calcium ions on the enzyme activity was also studied. In the usual test system, $MgSO_4 \cdot 7H_2O$ was added at a concentration of 0·015 *M*. For studying the effect of Ca^{2+} ions, $CaCl_2$-$2H_2O$ was added to the test system instead of $MgSO_4.7H_2O$. The final concentration of the Mg^{2+} or the Ca^{2+} ions was identical. The rest of the procedure with regard to incubation time and the method of assay of the DNase I activity was the same as described above.

A comparative study of the effect of *in vitro* addition of 6-thioguanine, amethopterin and nitrogen mustard [methyl-bis (beta-chloroethyl) amine hydrochloride] on DNase I activity from plasma, bovine pancreatic DNase I and exocellular DNase I of *P. chrysogenum*, was also made. Various agents were used at 2 mg. levels in a final volume of 3.7 ml. of the system. The other conditions such as *p*H, temperature and time of incubation were the same as described above.

Enzyme kinetic studies were conducted in order to determine the relative K_m value and the nature of the inhibition of exocellular DNase I activity of *P. chrysogenum* by nitrogen mustard. The procedure employed was similar to the one reported earlier[22]. Solution of nitrogen mustard was made in Tris buffer (0·1 *M*; *p*H 7·3) and was added at the level of 0·8 mg. in a total volume of 3·7 ml. The nature of inhibition of the enzyme was studied by plotting the reciprocals of the substrate concentrations *versus* the reciprocals of the enzyme concentrations, in the presence and in the absence of nitrogen mustard.

Results and Discussion

During preliminary investigations of the levels of exocellular DNase I of *P. chrysogenum* it was observed that culture filtrates obtained during early stages of fermentation contained high concentrations of acid-soluble deoxyribonucleotides. A systematic study was, therefore, carried out for determining acid-soluble nucleotides, DNase I activity and penicillin production from samples of the culture filtrates collected at different stages of the fermentation. The results of this study, presented in Fig. 1, show that the amount of acid-soluble deoxyribonucleotides in the culture filtrate is maximum at the end of the first day, i.e. during the stage of rapid multiplication of the organism and prior to the beginning of the phase of antibiotic production. During the phase of penicillin production, i.e. from second day onwards the acid-soluble deoxyribonucleotide content of the culture filtrates gradually decreased until the end of the fermentation cycle. It, therefore, appears that the acid-soluble deoxyribonucleotides are accumulated in the culture filtrate during early stages prior to the period of penicillin excretion and are possibly utilized by the organism during the active phase of penicillin production. Thus, it would be of interest to study the concentrations of both intracellular and exocellular deoxyribonucleotides at different

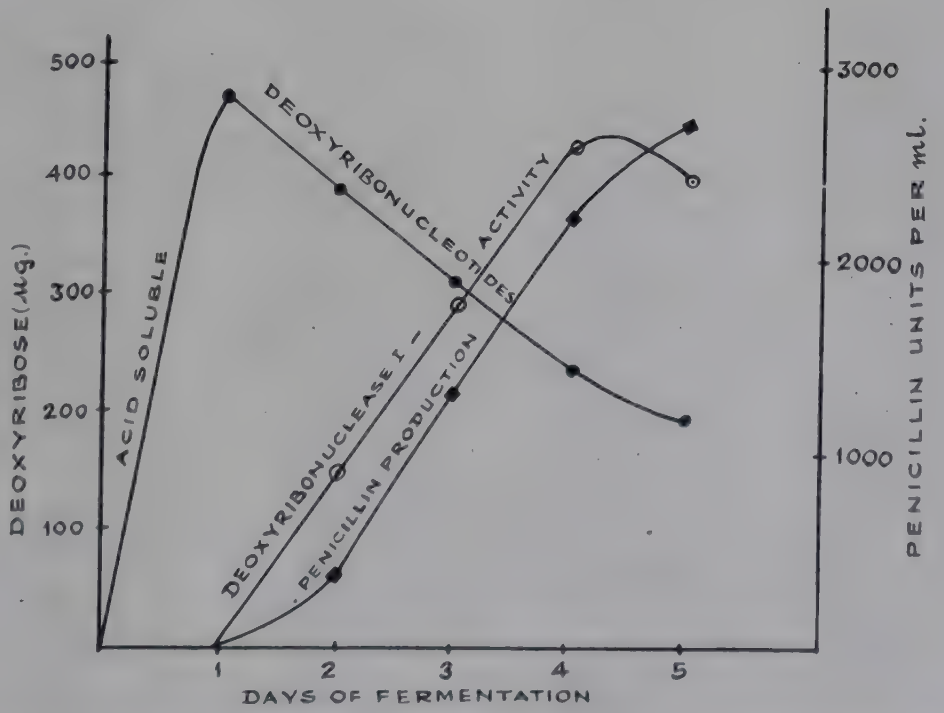

Fig. 1—Changes in the levels of DNase I, penicillin and acid-soluble deoxyribonucleotides in the culture filtrates of *P. chrysogenum* during penicillin fermentation (for details of the procedure and DNase I refer the text)

stages of fermentation and then attempt to correlate these changes to the yield of the antibiotic production. However, commensurate with mycelial growth, exocellular DNase I activity and the penicillin yield rapidly increased after the second day and continued to rise till the end of the fermentation at which time mycelial lysis sets in. The enzyme thus appears to be continuously excreted into the culture filtrate during the process of penicillin production. Subsequently the lysis of the mycelium sets in and a slight drop in the enzyme activity is observed at the end of the cycle. Since exocellular DNase I activity was maximum after 4 days fermentation, culture filtrates obtained from these samples were used for studying the properties of the enzyme.

Properties of the enzyme. The main object of this investigation was to make a comparative study of the properties of DNases I obtained from various sources. The enzyme activity measurements in the following experiments were carried out according to the procedure described above. Incubations were carried out at 45°C. for 2 hr.

The results presented in Table 1 show that DNase I activity from culture filtrate of *P. chrysogenum* or from bovine pancreas is destroyed by heating at 100°C. for 30 min. at *p*H 9·0, 2·8 and 7·0; however, the plasma DNase I of Cohn fraction VI-2 is stable to heat at the three *p*Hs as reported earlier[22]. DNase I of *Micrococcus pyogenes* reported by Cunningham *et al.*[17] is also heat stable under various *p*H conditions. However, this latter enzyme differs from the plasma enzyme or the DNase I from *P. chrysogenum* or the bovine pancreatic DNase I in that it requires calcium instead of magnesium as cationic activator. DNase I activity of *P. chrysogenum* is completely inhibited by the addition of Ca^{2+} instead of Mg^{2+} in the test system.

The results on the effect of nitrogen mustard, 6-thioguanine or amethopterin on the DNase I activities from different sources are presented in Table 2. Nitrogen mustard and amethopterin inhibited DNase I from all the three sources. However, 6-thioguanine inhibited DNase I from

TABLE 1—HEAT STABILITY OF DNAASES I FROM *P. chrysogenum* CULTURE FILTRATE, BOVINE PANCREAS, OR HUMAN PLASMA COHN FRACTION VI-2

Source of DNAase I	*Destruction of DNase I after heating in a boiling water-bath for 30 min. at		
	*p*H 9·0	*p*H 2·8	*p*H 7·0
P. chrysogenum culture filtrate	+	+	+
Bovine pancreas[22]	+	+	+
Human plasma Cohn fraction VI-2[22]	—	—	—

*For details of the treatment and method of DNase I assay refer the text. Plus sign indicates complete destruction of enzyme activity while minus sign indicates no destruction.

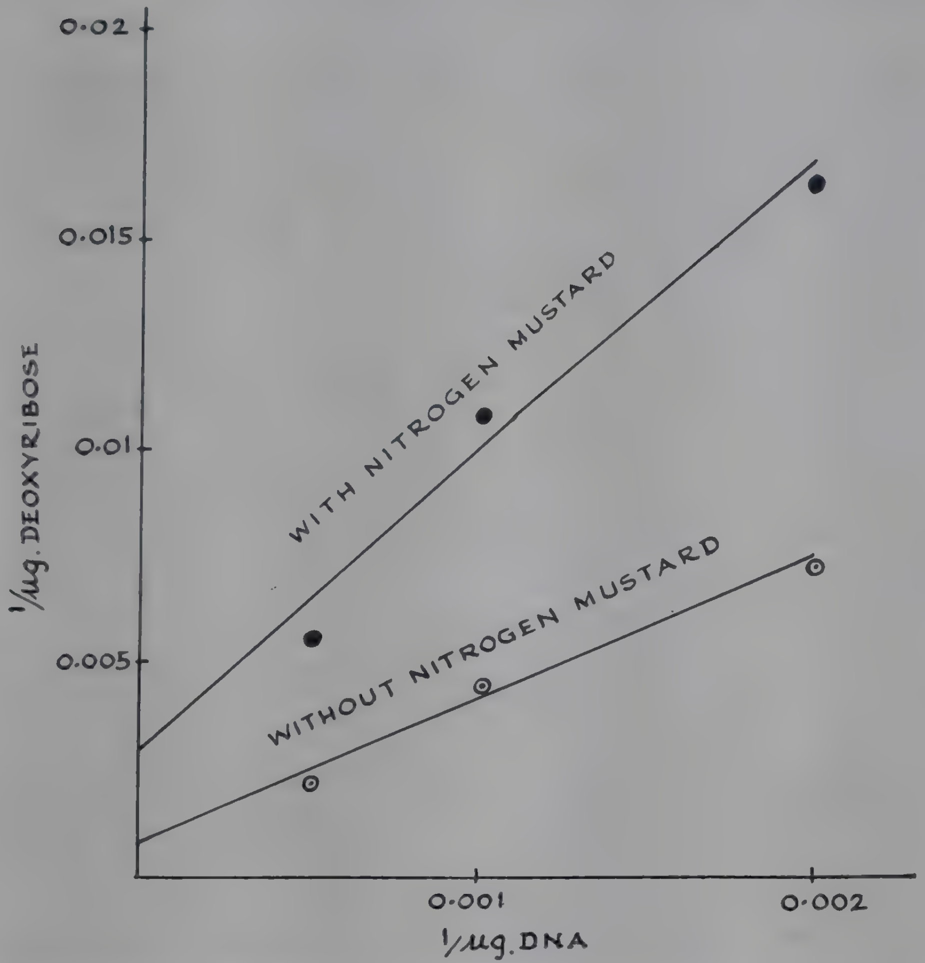

Fig. 2—Kinetic study of the *in vitro* inhibitory effect of nitrogen mustard [methyl-bis (beta-chloroethyl)-amine hydrochloride] on DNase I of culture filtrate from *P. chrysogenum* (For details of the procedure and DNase I refer the text)

P. chrysogenum culture filtrates or from bovine pancreas but not the DNase I of plasma Cohn fraction VI-2. In Fig. 2 are presented the results of the kinetic studies on the inhibition of DNase I of *P. chrysogenum* culture filtrates by *in vitro* addition of nitrogen mustard. The plot of the reciprocal of DNase I activity in the presence or in the absence of nitrogen mustard gives two separate straight lines with different slopes and intercepts. According to the mathematical formulation of Lineweaver and Burk[25], this would indicate that nitrogen mustard inhibits the DNase I of *P. chrysogenum* culture filtrates in a non-competitive manner. *In vitro* addition of nitrogen mustard also inhibits non-competitively DNase I activity of bovine pancreas or DNase I activity of plasma or DNase I of Cohn fraction VI[22]. The relative K_m values were also calculated for the DNase I from the three sources by plotting 1/v *versus* 1/s curves. The results indicate that the relative K_m value of DNase I from *P. chrysogenum* culture filtrate is very close to that of the K_m value reported[22] for plasma DNase I. However, the reported K_m value for the bovine pancreatic DNase I is 10 times higher than the K_m value for the DNase I of *P. chrysogenum.*

Further studies on the nature of cleavage of DNA by DNase I of *P. chrysogenum* culture filtrate or by DNase I human plasma are in progress.

TABLE 2—EFFECT OF NITROGEN MUSTARD OR ANTIMETABOLITES ON DNASES I FROM *P. chrysogenum* CULTURE FILTRATE, BOVINE PANCREAS OR HUMAN PLASMA COHN FRACTION VI-2

Source of DNase I	*Inhibition by		
	Nitrogen mustard %	6-Thioguanine %	Amethopterin %
P. chrysogenum culture filtrate	43	15	43
Bovine pancreas[22]	14	19	16
Human plasma Cohn fraction VI-2[22]	21	00	32

*For details of treatment and method of DNase I assay refer the text.

SUMMARY

Daily changes in the levels of exocellular DNase I of *Penicillium chrysogenum* HA 10 were studied during penicillin fermentation. The organism was grown under submerged conditions in a chemically defined medium and the fermentation was continued for a five-day period. Penicillin yield and exocellular DNase I activity increased after the second day of fermentation and continued to rise till the end of the fermentation at which time mycelia started lysing. In contrast to this, the level of acid-soluble deoxyribonucleotides present in the culture filtrate was highest at the end of the first day of fermentation and gradually decreased from second day onwards till the end of the fermentation. The results appear to suggest that the acid-soluble deoxyribonucleotides, which were formed in a very high concentration prior to the phase of penicillin production, are continuously utilized by the organism during the phase of penicillin production.

The properties of exocellular DNase I of *P. chrysogenum* were compared with those of DNases I from human plasma and bovine pancreas. The *P. chrysogenum* DNase I is similar to bovine pancreatic DNase I with regard to its stability to *p*H and heat, and also with regard to *in vitro* inhibition by nitrogen mustard [methyl-bis (beta-chloroethyl)-amine hydrochloride], 6-thioguanine and amethopterin. The relative K_m value of the exocellular DNase I of *P. chrysogenum* is very close to the human plasma DNase I and is one-tenth the K_m value obtained for the bovine pancreatic DNase I.

Enzyme kinetic study on the nature of the *in vitro* inhibition of exocellular DNase I of *P. chrysogenum* by nitrogen mustard indicated that the enzyme is non-competitively inhibited by the nitrogen mustard.

ACKNOWLEDGEMENT

The authors wish to thank Dr M. J. Thirumalachar for the culture of *Penicillium chrysogenum* HA 10 and also for his encouragement during the course of the work.

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Characteristics of a Protein Specified by a Regulator Gene

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Connecticut, U.S.A.

Synthesis of the enzyme alkaline phosphatase by *Escherichia coli* is repressed under certain environmental and genetic conditions. In the standard strain, repression occurs when the growth medium contains a high concentration of orthophosphate[1–3]. This repression by orthophosphate can be prevented by mutations, located in either of two regions of the bacterial chromosome, which produce phosphatase-constitutive strains. One region (*R*1) is closely linked to the structural gene for the enzyme, and the other (*R*2) is located farther away[4]. The *R*2 region appears to consist of two functional units[5], *R*2*a* and *R*2*b*.

Both the *R*1 and *R*2 classes of phosphatase-constitutive mutations are recessive to the standard alleles in bacterial heterozygotes, indicating that the products specified by the standard genotypes of the two regions are required for the formation of a repressor for alkaline phosphatase[4]. By this genetic criterion, both regions are regulator genes[6]. The designation of a regulator gene does not necessarily imply that the gene product is itself a repressor; it might instead be involved in a reaction required for the formation of a repressor[7,8].

There is evidence that the products specified by the *R*1 and *R*2 regions are proteins[9], from the genetic observation that some of the phosphatase-constitutive mutations in both regions are suppressible by a particular suppressor gene. Other indirect evidence that the *R*2 region specifies a protein has been obtained from physiological studies with a temperature-sensitive *R*2 phosphatase-constitutive mutant[10].

The overall reaction leading to the formation of a repressor for alkaline phosphatase can be summarized as follows:

$$\begin{tikzcd}
R1\text{ gene} \arrow[d] & R2a\text{ gene} \arrow[d] & R2b\text{ gene} \arrow[d] & \\
R1\text{ protein} + & R2a\text{ protein} + & R2b\text{ protein} + & \text{orthophosphate repressor}
\end{tikzcd}$$

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Thus, there are at least three functions, controlled by the three regulator genes, *R*1, *R2a* and *R2b*, that are required for repression of alkaline phosphatase synthesis in *Esch. coli*. A complete understanding of the mechanism of regulation of this enzyme depends on the identification of the products of each regulator gene and the elucidation of their respective functions.

As a first step towards this goal, we have succeeded recently in isolating the *R2a* protein specified by the *R2a* regulator gene[5]. The procedure used to identify this protein involves a comparison of the proteins produced by two strains, one having a functional *R2a* gene and the other a defective *R2a* gene (i.e. an $R2a^-$ constitutive mutant). The results (Figs. 1 and 2) show that one of the protein bands is lost as a result of an $R2a^-$ mutation. Mutations in the other regulator genes, *R*1 and *R2b*, do not affect this protein.

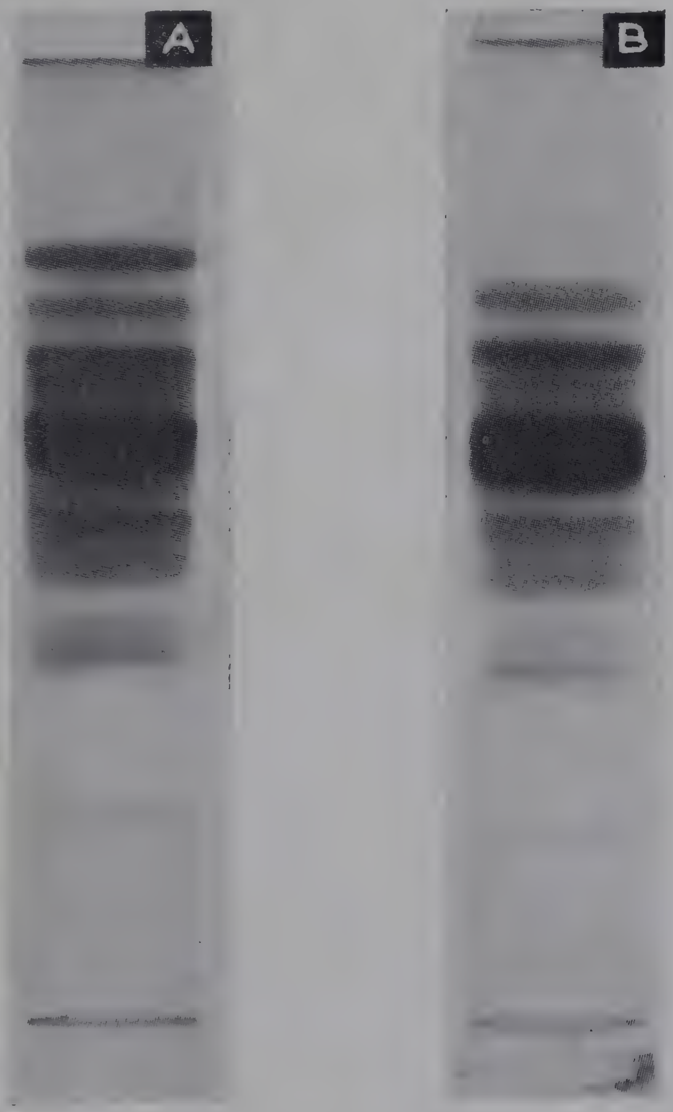

FIG. 1—IDENTIFICATION OF THE *R2a* REGULATOR PROTEIN BY DISC GEL ELECTROPHORESIS: (A) An extract prepared from an $R1^-$ constitutive mutant, in which the regulator protein appears as the slowest moving band (towards the top of the gel). (B) An extract prepared from an $R2a^-$ constitutive mutant, in which the regulator protein band is missing (For experimental details, see ref. 5)

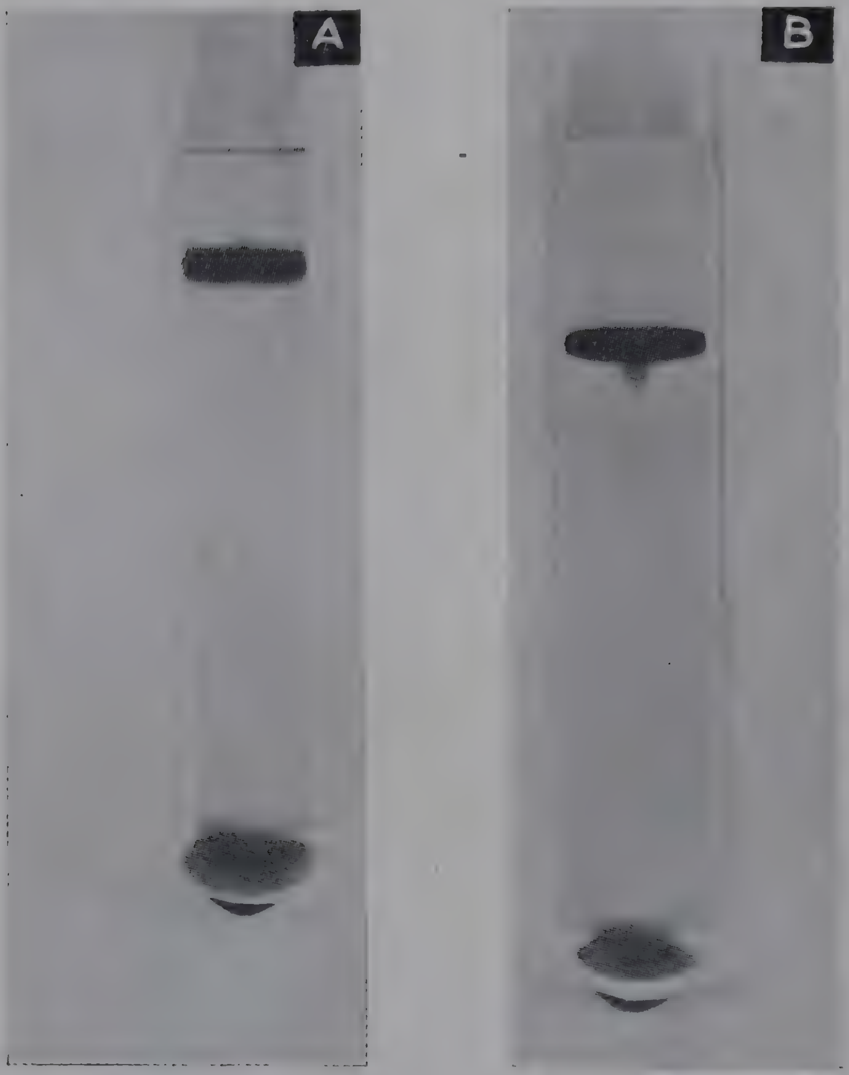

Fig. 2—Disc gel electrophoresis of purified *R2a* regulator protein: (A) Electrophoresis buffer at *p*H 8·3. (B) Electrophoresis buffer at *p*H 4·5 (For experimental details, see ref. 5)

Analyses of the *R2a*⁻ protein after purification indicate a sedimentation constant of 3·2 S and an isoelectric point of 5·7. The protein appears, by chemical and immunological tests, to consist of a single molecular species[5].

Definitive evidence that the structure of the *R2a* protein is specified by the *R2a*⁻ regulator gene has been obtained by the identification of an electrophoretically altered form of the protein produced by a revertant of an *R2a*⁻ constitutive mutant, as shown in Fig. 3. This indicates that a reverse mutation within the *R2a* region (one that restores activity to a defective *R2a*⁻ gene) can result in an electrophoretic change in the protein, presumably due to an amino acid substitution at some position in the polypeptide chain.

A new aspect of the regulatory mechanism for alkaline phosphatase has emerged from studies on the *R2a* protein. It has been found that formation of the *R2a* protein is repressed under the same genetic and environmental conditions that cause repression of alkaline phosphatase, namely the presence of a functional set of *R*1, *R2a* and *R2b* regulator genes and a high concentration of orthophosphate in the medium. The existence of common requirements for repression of alkaline phosphatase and *R2a* protein suggests that both are regulated by the same repressor. This observation may provide an answer to an outstanding question about regulation: How is the activity of a regulator gene itself regulated ? The behaviour of the *R2a* gene indicates that a repressor, whose formation is controlled by a regulator gene, can act to repress the function of that gene.

Although alkaline phosphatase and *R2a* protein share a common regulatory mechanism, the maximum amounts of the two proteins produced under conditions of derepression differ markedly; alkaline phosphatase represents 6% of the total cell protein[3] and the *R2a* protein about 0·1%.

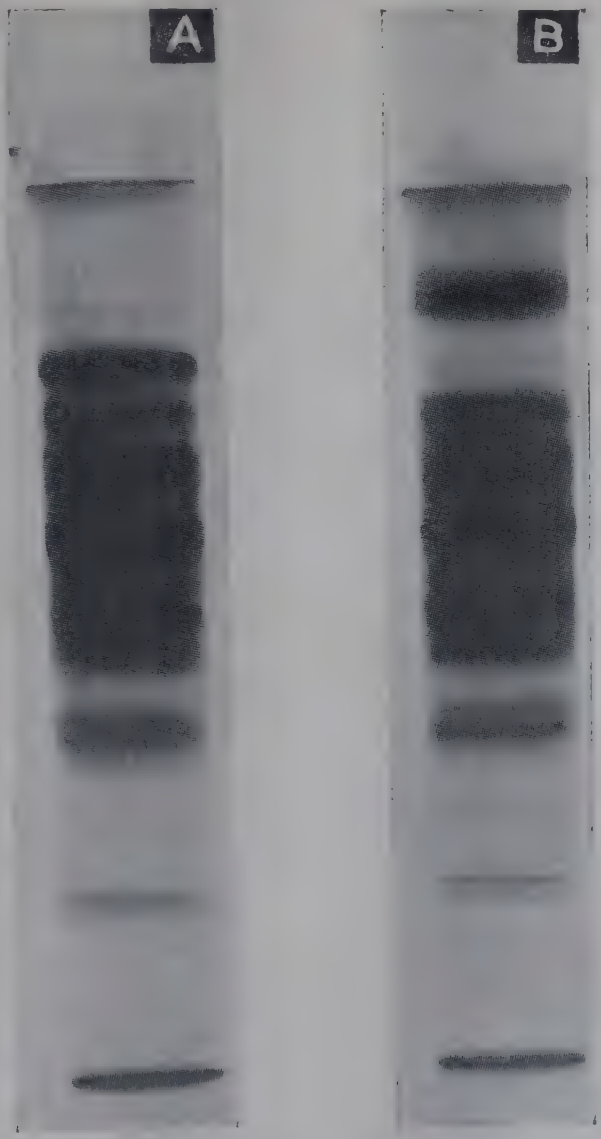

FIG. 3—FORMATION OF ELECTROPHORETICALLY ALTERED *R2a* PROTEIN BY A REVERTANT STRAIN: (A) Disc electrophoresis pattern from a normal revertant, in which the *R2a* band is in the standard position (the slowest moving band, cf. Fig. 1). (B) Corresponding disc pattern from an altered revertant, in which the *R2a* protein has an increased net positive charge (For experimental details, see ref. 5)

The rate of synthesis of a particular protein must depend, therefore, not only on the degree of repression but also on other genespecific factors.

It is clear that the *R2a* protein has some essential role in the formation of a repressor for alkaline phosphatase. What is the nature of this role ? The *R2a* protein itself cannot act as a repressor, since it is produced under conditions for derepression of alkaline phosphatase. It might be a structural component of a repressor; or, it might participate in a reaction required for the formation of a repressor. Further studies on the properties of the *R2a* protein should help to answer this question.

SUMMARY

The product specified by one of the three regulator genes for alkaline phosphatase, the *R2a* gene has been identified and purified. It is a protein which is essential for the repression of alkaline phosphatase synthesis, but is not the alkaline phosphatase repressor, *per se*.

Formation of the *R2a* protein is repressed under the same genetic and environmental conditions that are required for the repression of alkaline phosphatase, suggesting that the same repressor regulates both proteins.

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Fluorinated Pyrimidines: Biochemically and Clinically Useful Antimetabolites

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The successful concept for the fluorinated pyrimidine antimetabolites as cancer chemotherapeutic drugs arose as a consequence of the observation in 1954 by Rutman, Cantarow and Paschkis[1]. These workers demonstrated that a chemically induced rat hepatoma utilized uracil for nucleic acid biosynthesis to a greater extent than did the normal liver. In normal tissues it had been considered that orotic acid (Fig. 1) is the primary precursor of the nucleic acid pyrimidines, and uracil is used only to a slight extent. We were able to confirm and extend those observations and show that a number of transplanted tumors utilized uracil to a considerably greater extent than they did orotic acid[2]. Actually, some of this work was done by the Chairman of this Symposium, Dr P. M. Bhargava, in my laboratory. Because of this enhanced utilization of uracil we wished to design an antimetabolite that would resemble uracil as closely as possible in structure, but differ in one significant respect. But before considering this, it is necessary to review the fact that uracil is found only in RNA and not in DNA, and that thymine (Fig. 1) an essential building block of DNA, is made in cells by the attachment of one carbon to the preformed pyrimidine ring of a derivative of uracil at carbon-5. We will consider this in more detail later.

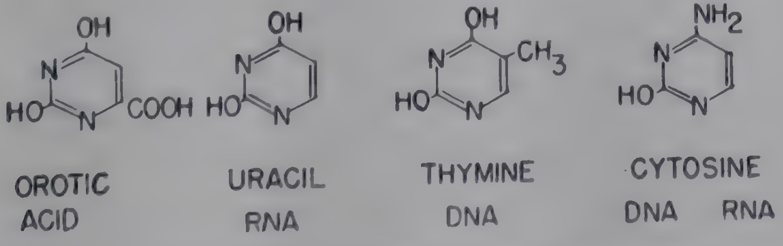


Fig. 1—Structures of nucleic acid pyrimidines

The alteration in the uracil molecule considered to be most promising was to substitute a fluorine atom for a hydrogen atom, because such a substitution converts non-toxic acetic acid to fluoroacetic acid, used commercially as a rat poison. There are two loci in the uracil molecule where such a substitution could be made, on carbons 5 or 6. The former was chosen for two reasons: one that it seemed more amenable to synthesis, and two, the location of a fluorine atom on the 5 position might be expected to block the attachment of the carbon atom that forms the methyl group of thymine. The desired structure, 5-fluorouracil is shown in Fig. 2, which also demonstrates the close correspondence in size between the hydrogen and fluorine atoms; chlorine is very much larger. The successful synthesis of 5-fluorouracil (FU) is shown in Fig. 3, and was carried out in a collaboration between myself and Dr Robert Duschinsky of Hoffmann-LaRoche, Inc[3]. The compound turned out to have considerable activity at inhibiting transplanted tumors in mice and rats[4,5]. In attempts to improve the therapeutic effectiveness of FU, the corresponding ribonucleoside, FUR, and

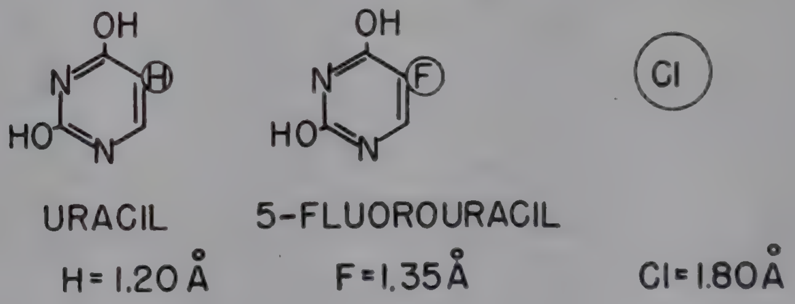

OH
N
H
HO
N
OH
N
F
HO
N
Cl
URACIL
5-FLUOROURACIL
H = 1.20 Å
F = 1.35 Å
Cl = 1.80 Å

FIG. 2—STRUCTURE OF 5-FLUOROURACIL

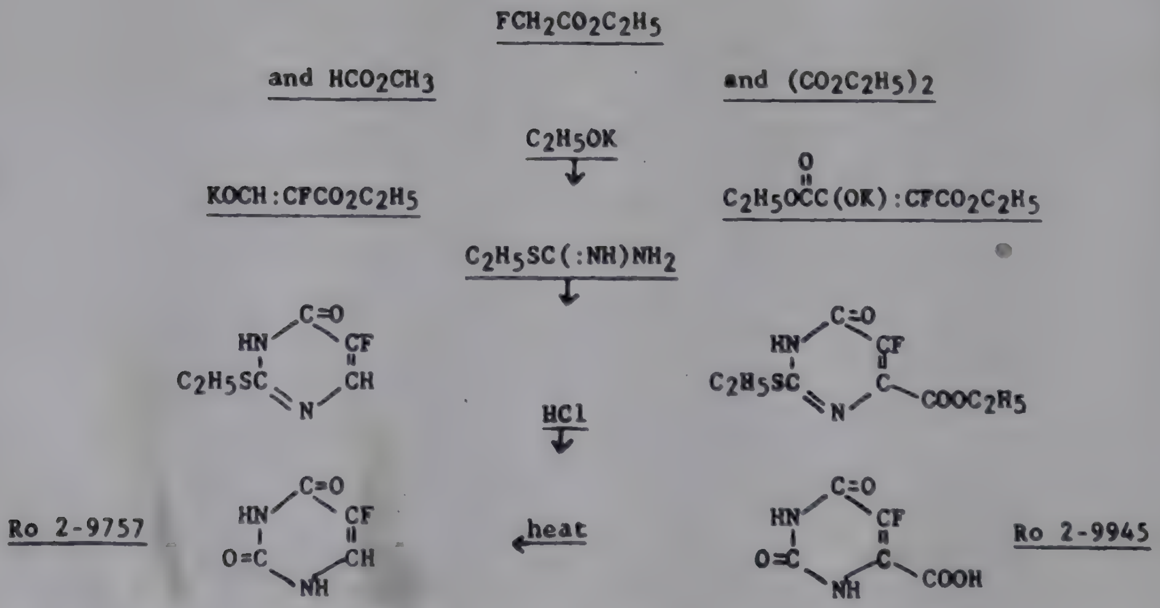
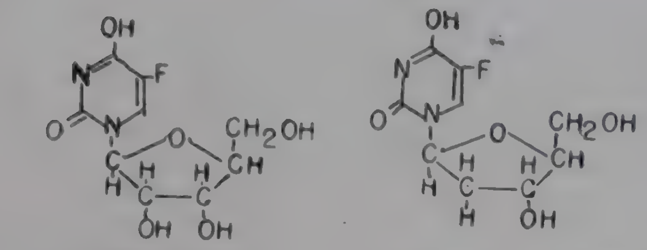

FCH2CO2C2H5
and HCO2CH3
and (CO2C2H5)2
C2H5OK
KOCH:CFCO2C2H5
C2H5OCC(OK):CFCO2C2H5
C2H5SC(:NH)NH2
C=O
HN
CF
C2H5SC
CH
N
C=O
HN
CF
C2H5SC
C
COOC2H5
N
HCl
Ro 2-9757
C=O
HN
CF
O=C
CH
NH
heat
C=O
HN
CF
O=C
C
COOH
NH
Ro 2-9945

FIG. 3—SYNTHESIS OF 5-FLUOROURACIL



5-FLUOROURIDINE
FUR
RO 5-0360/2

5-FLUORO-2'-DEOXYURIDINE
FUDR
RO 5-0338

FIG. 4—STRUCTURES OF THE NUCLEOSIDES OF 5-FLUOROURACIL

deoxyribonucleoside, FUDR, were prepared (Fig. 4). FUR was considerably more toxic than FU and less effective against most tumors and can hardly be considered to be a better drug[6]. However, FUDR did have better anti-tumor activity with less toxicity than FU, and hence is an improvement[6].

At this point we became interested in studying the biochemical mechanisms of action of this series of compounds, and another of my post-doctoral fellows from India, Dr N. K. Chaudhuri, synthesized ^{14}C-labelled FU and studied its metabolism. We found that there was incorporation of FU as such into the RNA, but not the DNA, of mouse tumors and normal tissues[7]. An ion-exchange chromatogram of the alkaline hydrolysate of RNA is shown in Fig. 5, and all the radioactivity was associated with a peak identified as fluorouridylic acid[7].

We also wished to determine whether FU and FUDR do in fact inhibit the attachment of the methyl group to the ring system of uracil to give thymine. Formate-^{14}C is a specific precursor of the methyl group of thymine, so we incubated suspensions of Ehrlich ascites tumor cells with formate in the presence and absence of various concenrtations of fluorinated pyrimidines, and measured the specific radioactivity of the DNA thymine. The results are shown in Fig. 6, and demonstrate that these compounds do, as predicted, inhibit this biosynthetic pathway[8].

The enzyme that carries out this methylation is called thymidylate synthetase and as shown in Fig. 7 requires a tetrahydrofolic acid coenzyme and attaches the one carbon from methylene tetrahydrofolate to the 5 -carbon of deoxyuridylate to give thymidylate (TDRP). The hydrogens from the ring of the tetrahydrofolic acid are donated to the methyl group, and a stoichiometric amount of dihydrofolic acid is produced. The inhibition of this enzyme obtained from phage-infected *Esch. coli* by the nucleotide, 5-fluoro-2′-deoxyuridine-5′-monophosphate (FUDRP), was first demonstrated by Cohen *et al.*[9] We have confirmed and extended this work to mammalian systems and have studied the kinetics of this inhibition[10], and as shown in Fig. 8 the inhibition by FUDRP is competitive, whether the nucleotide inhibitor is added simultaneously with the substrate or after preincubation, although the ratio of K_m/K_i is altered.

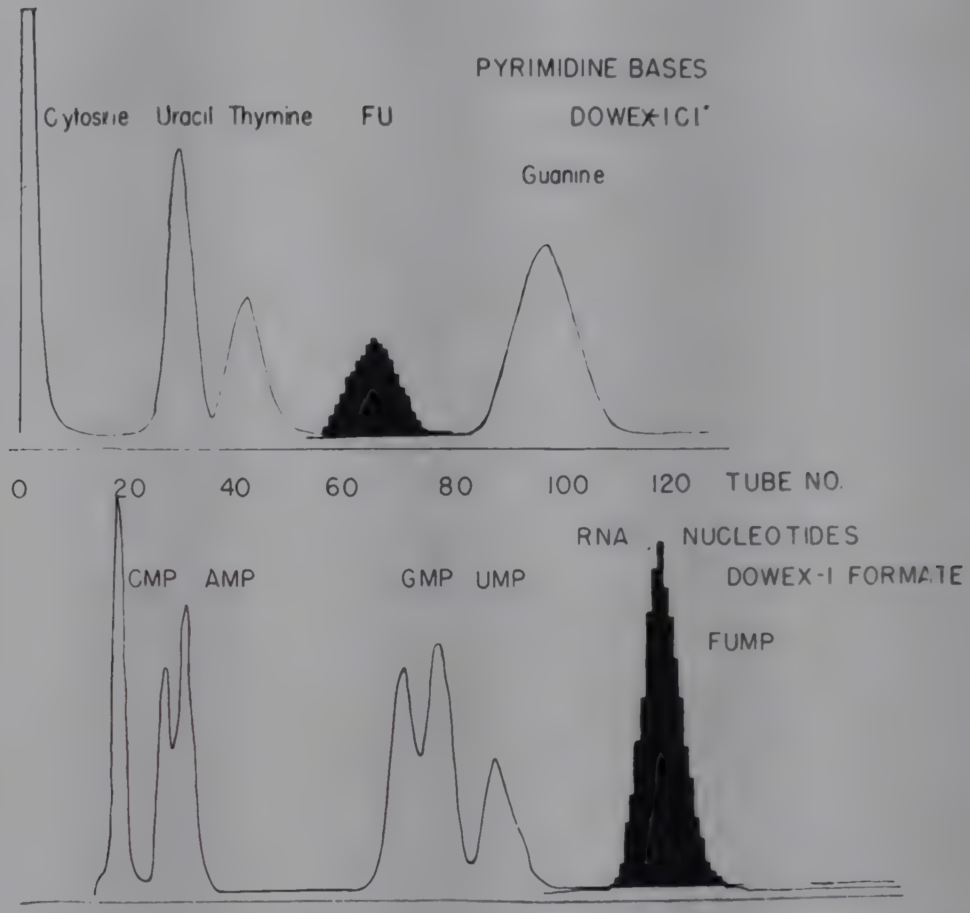


FIG. 5—CHROMATOGRAM ON DOWEX-1-FORMATE OF ALKALINE HYDROLYSATE OF RNA. THE SOLID PEAK REPRESENTS THE RADIOACTIVITY

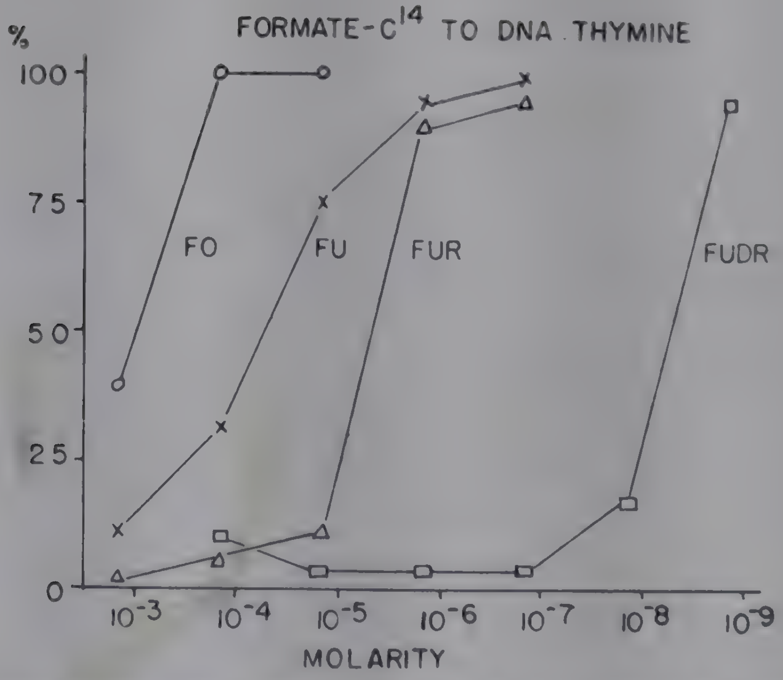


FIG. 6—EFFECT OF FLUORINATED PYRIMIDINES ON THE INCORPORATION OF FORMATE-^{14}C INTO DNA THYMINE OF SUSPENSIONS OF EHRLICH ASCITES CARCINOMA CELLS[8]

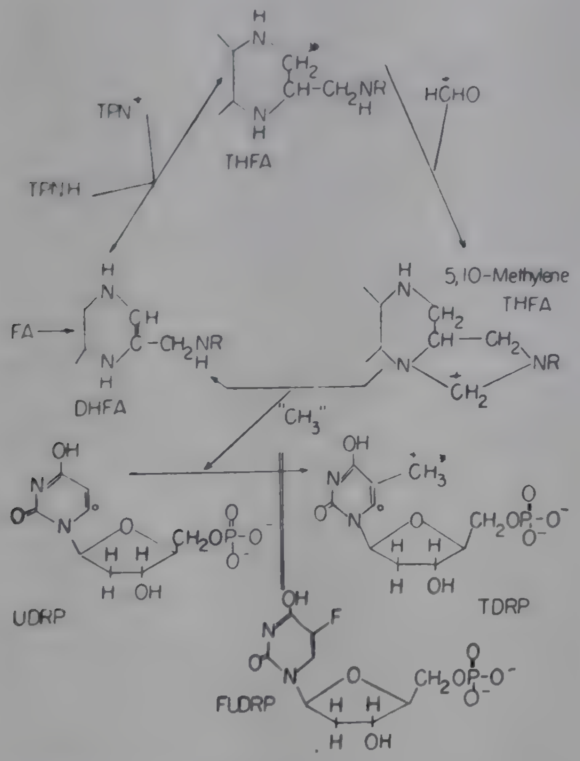
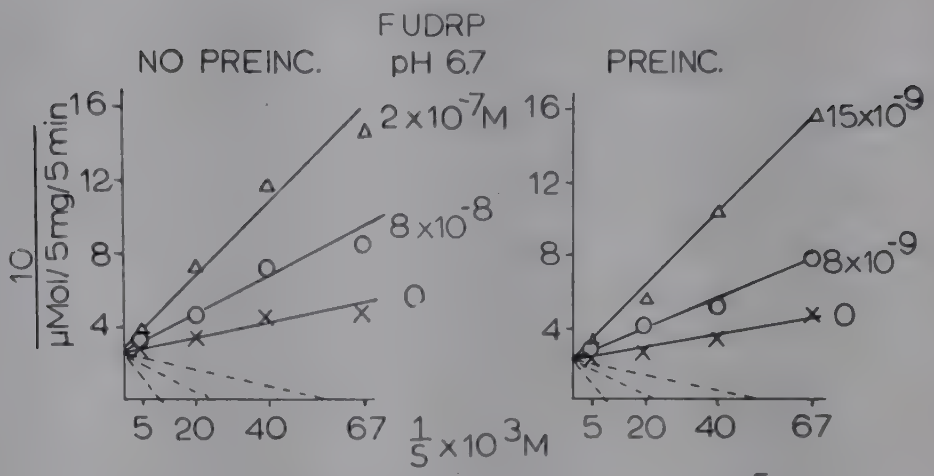


FIG. 7—SCHEME REPRESENTING METHYLATION BY THYMIDYLATE SYNTHETASE[10]

The metabolic degradation of FU and FUDR has been studied by two of my post-doctoral fellows from India, Drs N. K. Chaudhuri and K. L. Mukherjee[11], and the scheme of degradation as well as anabolism is shown in Fig. 9, which summarizes what we know about the metabolism of this series of compounds. There has been considerable biochemical work by many other workers in a variety of systems, including viral ones. This I have reviewed elsewhere, and a discussion is beyond the scope of this lecture[12]. These compounds are useful in the palliative treatment of patients with various far advanced cancers. The chemotherapeutic aspects have been reviewed[13], and will be dealt with in one of the evening lectures at this Symposium.

I now wish to turn my attention to another series of fluorinated pyrimidine antimetabolites that we have recently developed. Because of the similarity in size between fluorine atoms and hydrogen atoms, it seemed likely that thymine, altered so that the methyl group would be replaced by a trifluoromethyl group (Fig. 10), might be incorporated into DNA, replacing thymine, as is bromouracil. 5-Trifluoromethyluracil ("trifluorothymine," F_3T) was synthesized in my laboratory and converted enzymatically to the corresponding nucleoside, F_3TDR[14]. One important consequence of this alteration in the molecule is its greatly increased acidity (Fig. 10), which will be referred to again. The scheme of the synthesis is shown in Fig. 11.

At first we had only a small amount of the free base, F_3T, and in an attempt to learn something of its biochemical behaviour we studied it in a



	NO PREINC.	PREINC.
K_m	1.3×10^{-5}M	1.5×10^{-5}M
K_i	5.2×10^{-8}M	3.6×10^{-9}M
$\frac{K_m}{K_i}$	254	4220

FIG. 8—INHIBITION OF THYMIDYLATE SYNTHETASE BY FUDRP. LINEWEAVER-BURK PLOT[10]

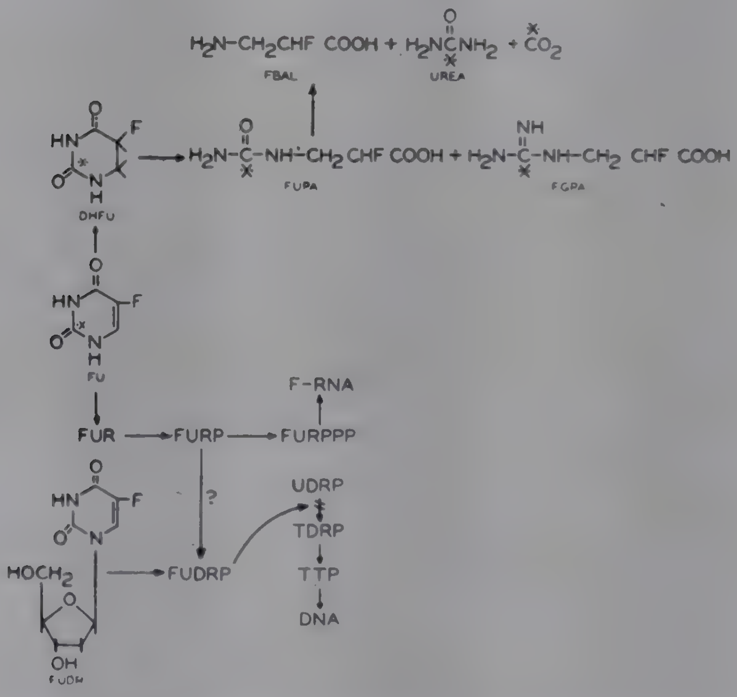

H_2N-CH_2CHF COOH + H_2NCNH_2 + CO_2
FBAL
UREA
DHFU
FUPA
FGPA
FU
FUR
FURP
FURPPP
F-RNA
UDRP
TDRP
TTP
DNA
FUDRP
?
HOCH$_2$
OH
FUDR

FIG. 9—CATABOLIC AND ANABOLIC REACTIONS OF 5-FLUOROURACIL AND DERIVATIVES

thymine-requiring mutant of *Esch. coli* 15T. Here we got our first surprise, because, as shown in Fig. 12, F_3T does not support the growth of this organism in the absence of thymine[15,16]. However, inhibition was produced in the presence of limiting amounts of thymine[16] (Fig. 12). 5-Bromouracil is incorporated into DNA and as a consequence is mutagenic to bacteriophage T4. We have studied the effects of F_3T and F_3TDR on the induction of forward mutations in this bacteriophage. As can be seen from Fig. 13 these

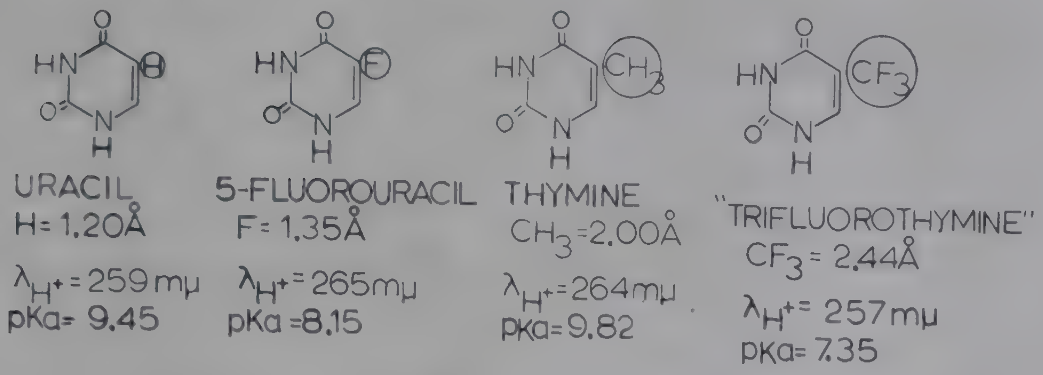

URACIL
H= 1.20Å
λH+ = 259 mμ
pKa= 9.45
5-FLUOROURACIL
F= 1.35Å
λH+ = 265 mμ
pKa = 8.15
THYMINE
CH3 = 2.00Å
λH+ = 264 mμ
pKa= 9.82
"TRIFLUOROTHYMINE"
CF3 = 2.44Å
λH+ = 257 mμ
pKa= 7.35

Fig. 10—Structures of pyrimidines and their fluorinated analogs

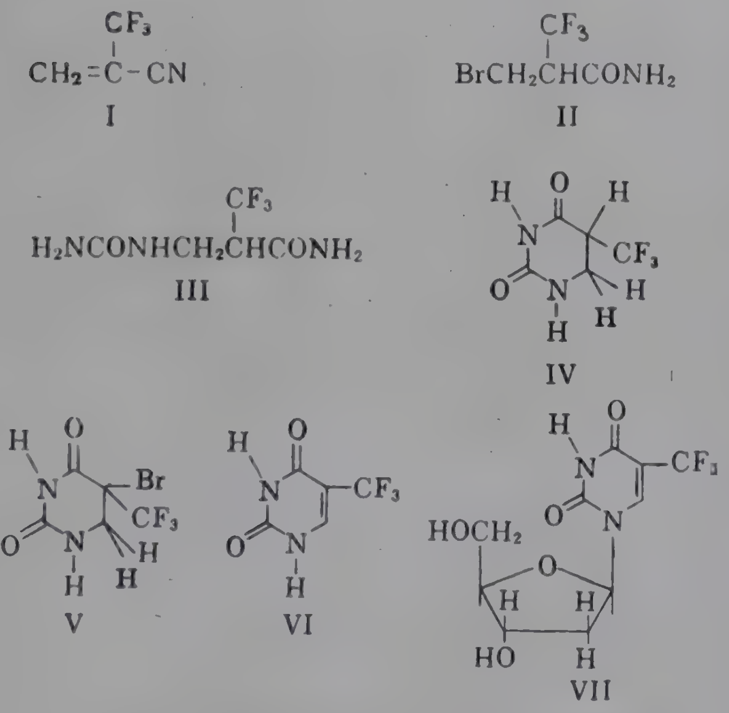

I
II
III
IV
V
VI
VII

Fig. 11—Synthesis of F_3T and F_3TDR[14]

compounds are mutagenic at the r locus, with activities comparable to the brominated pyrimidines[16]. A more specific test of mutational activity is the induction of reversions of point mutants of bacteriophages to the wild-type, and the compounds also demonstrated activity in this system[16].

Because of this mutagenic activity we inferred that F_3TDR was incorporated into DNA, and this was tested more directly. A large amount of bacteriophage T4 was grown for a short time in the presence of $F_3TDR\text{-}2\text{-}^{14}C$, the phages were purified, and the DNA was isolated. It was then centrifuged in a density gradient of cesium chloride, and the run in the analytical centrifuge is shown in Fig. 14. Here it can be seen that the DNA has a higher density than that of the normal phage, which showed that incorporation did take place into all the DNA that was replicated during this period[16].

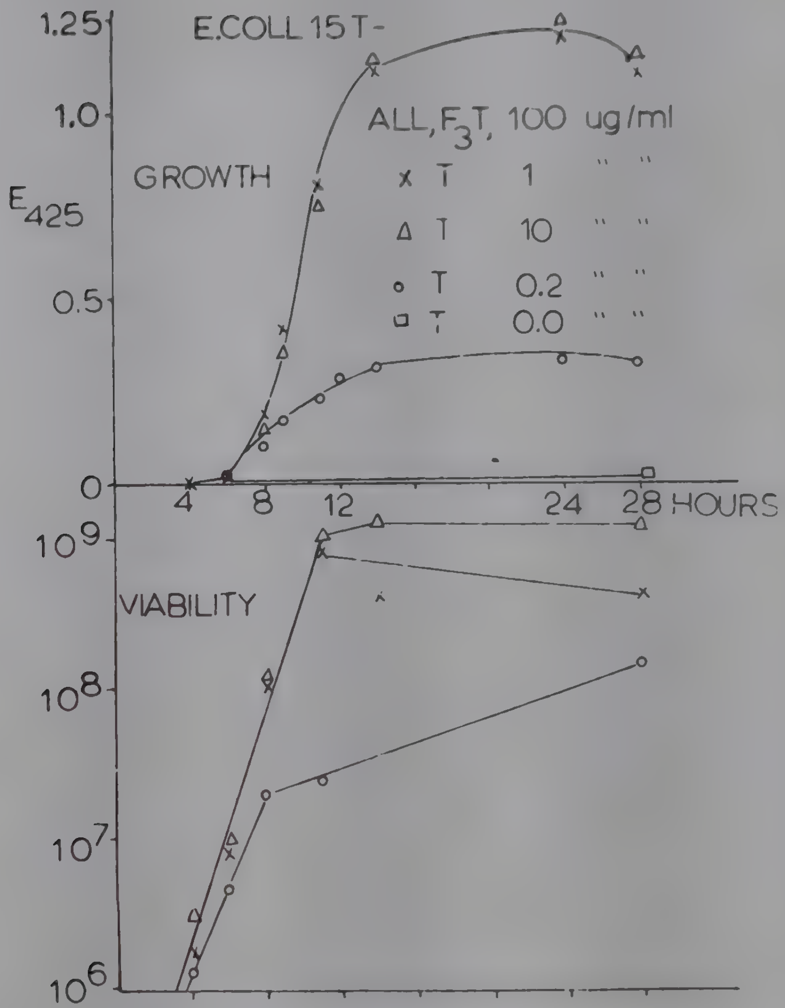


FIG. 12—EFFECT OF F_3T ON THE GROWTH OF *Esch. coli* 15 T[16]

Broth Medium

Analog 5-Substituted	Input Multiplicity	Conc. mM.	F_s^r r-Frequency Spontaneous $\times 10^{-4}$	F_i^r r-Frequency Induced $\times 10^{-3}$	F_i^r / F_s^r	Titer Increase Control	Titer Increase Treated	Ratio
BrU	0.08	2.78	2.5	0.84	3	2220	2160	1
F_3T	0.08	2.78	2.5	44.9	18	2220	4	564
F_3T	1.3	2.78	3.6	1.9	5	95	1.7	56
BrUDR	0.08	2.78	2.5	13.1	52	2220	388	6
F_3TDR	0.08	2.78	2.5	14.9	60	2220	635	4
F_3TDR	0.81	2.7	2.6	8.1	31	273	5	55

FIG. 13—INDUCTION OF FORWARD MUTATIONS AT THE LOCUS IN BACTERIOPHAGE T4[16]

When the sample was subjected to preparative density gradient centrifugation it was found (Fig. 15) that there was a complete correspondence between the optical density and the radioactivity, proving that F_3T is incorporated into DNA[16]. The amount of replacement of thymine amounted to 11%.

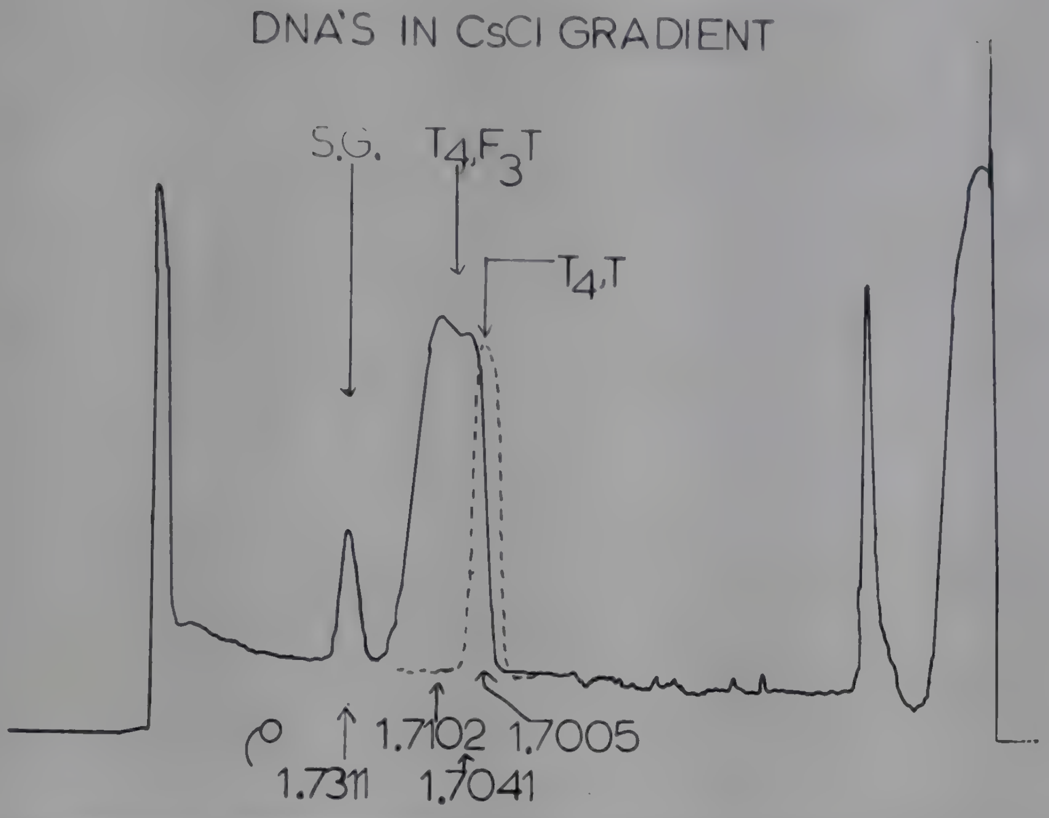


FIG. 14—ANALYTICAL CESIUM CHLORIDE DENSITY GRADIENT CENTRIFUGATION OF DNA FROM BACTERIOPHAGE T4 GROWN IN THE PRESENCE OF F_3TDR[12]

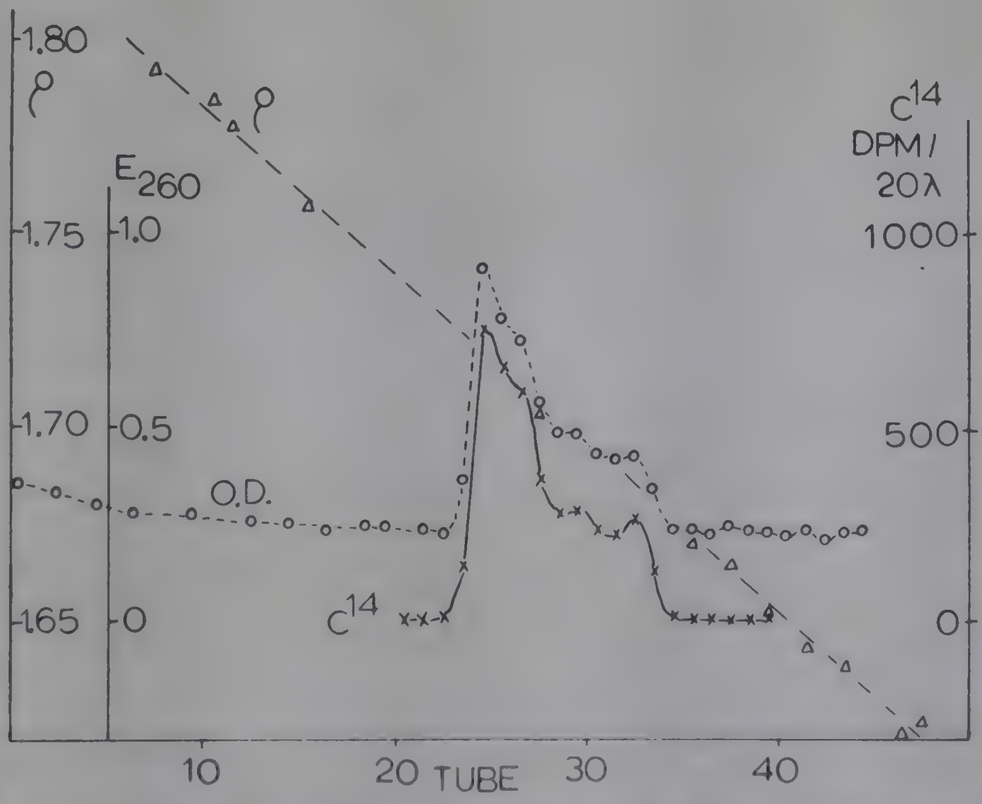


FIG. 15—PREPARATIVE CESIUM CHLORIDE DENSITY GRADIENT CENTRIFUGATION OF DNA FROM BACTERIOPHAGE T4 GROWN IN THE PRESENCE OF F_3TDR-2-^{14}C[16]

It was shown in the original chemical work that the trifluoromethyl group in this molecule is extraordinarily sensitive to very mild alkaline conditions, and F_3T is readily hydrolysed to 5-carboxyuracil (5-COOH-U)[14]. Thus, it became necessary to determine whether the compound incorporated into DNA had an intact CF_3 group, or whether it was converted to the carboxyl compound. Fortunately, F_3T is stable to acid hydrolytic conditions, and the chromatogram shown in Fig. 16 shows that all the compounds incorporated into DNA retained the trifluoromethyl group[16].

The increased acidity conferred upon the molecule by the inductive effect of the CF_3 group is demonstrated by the decreased *pKa* of the hydrogen at nitrogen-3. Since this hydrogen participates in the hydrogen binding in the double helical structure of DNA, we would expect that substitution of F_3T for thymine in the DNA would lead to an altered stability of the bonds holding the two strands together. That this supposition is correct is shown by the lower melting temperature (Fig. 17) of the DNA containing the analog, as compared to normal T4 phage DNA[16].

In collaboration with Prof. W. Szybalski of this laboratory, studies have been carried out on the effects of F_3TDR in human bone marrow cells grown in culture. It is extremely toxic to these cells; the toxicity is almost comparable to that of FUDR[17]. Further studies have shown that this toxicity can be reversed by thymidine, but not deoxycytidine or deoxyuridine[17]. In these mammalian cells, as well as in bacteriophage, F_3TDR is incorporated into DNA, the replacement amounting to about 5%[17]. As a consequence of this incorporation, the cells are sensitized to the lethal effects of ultraviolet and X-radiation[17].

Whereas the mutagenic and radiosensitizing effects of F_3TDR are explained by its incorporation into DNA, the failure to support the growth of a thymine-requiring mutant of *Esch. coli* and the great cytotoxicity to cells in culture cannot be readily explained on this basis. Accordingly, the effects of F_3T and F_3TDR were compared with FU and FUDR on the incorporation of formate-^{14}C into DNA thymine in suspensions of Ehrlich ascites cells, and it was found that these compounds do inhibit this incorporation almost as effectively as FU and FUDR[15], suggesting that the nucleotide, F_3TDRP, might also inhibit thymidylate synthetase. This would be somewhat surprising, since the metabolite that F_3TDRP resembles structurally is TDRP, the product of the enzyme and a very poor inhibitor of it. Therefore, F_3TDRP was prepared enzymatically (M. Y. Chang and C. Heidelberger, unpublished data) and has been studied in the thymidylate synthetase system.

When the inhibitor and the substrate were added simultaneously the inhibition was competitive, and the K_m/K_1 varied somewhat with the *p*H at which the assay was run (P. Reyes and C. Heidelberger, unpublished data). However, when the inhibitor was preincubated for 10 min. with the complete system before the addition of the substrate, non-competitive

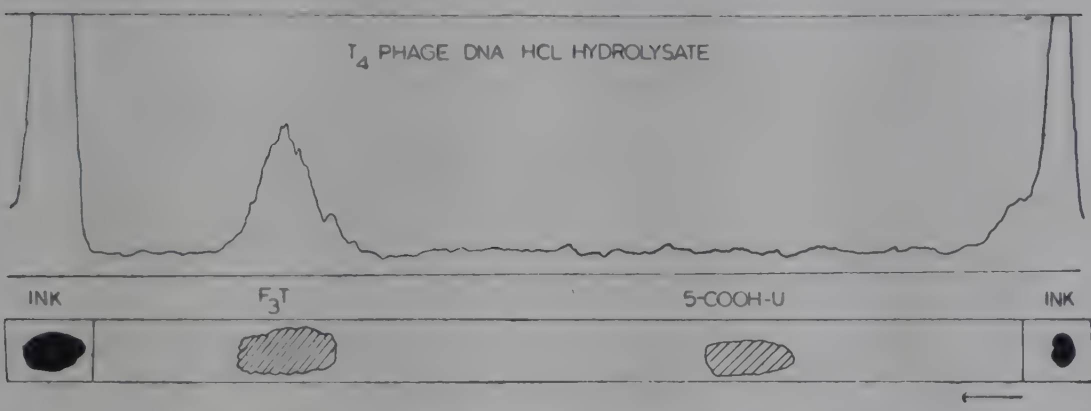


FIG. 16—PAPER CHROMATOGRAPH OF HYDROLYSATE OF BACTERIOPHAGE T4 DNA GROWN IN THE PRESENCE OF F_3TDR-2-^{14}C[16]

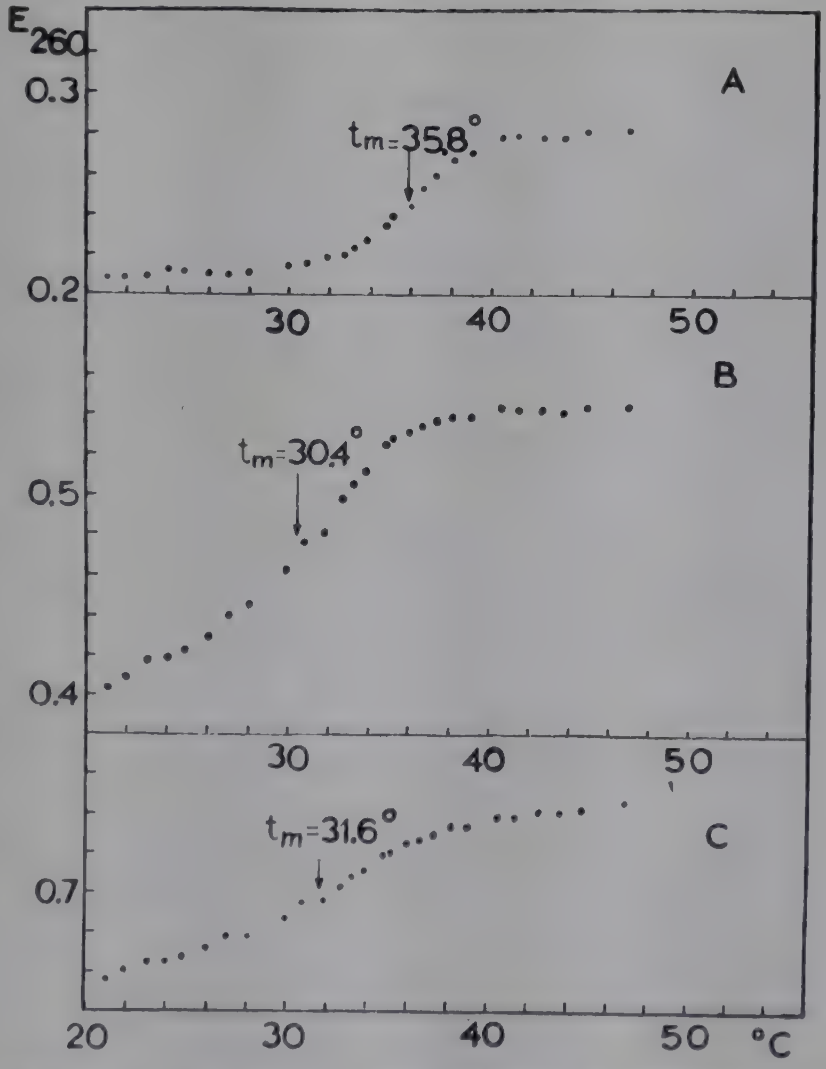


FIG. 17—THE MELTING TEMPERATURES OF THE DNA FROM BACTERIOPHAGE T4 IN 7*M* POTASSIUM PERCHLORATE: (A) DNA from normal phage and (B) DNA from phage grown in the presence of F_3TDR[16]

kinetics were obtained. At *p*H 6·7, the *p*H optimum for this enzyme, the kinetics are mixed. However, at *p*H 7·4, which is more physiological, the kinetics are purely non-competitive (P. Reyes and C. Heidelberger, unpublished data). The difference between the inhibitory effects of TDRP and F_3TDRP on this enzyme may again be explained by the greater acidity of the analog, suggesting that nitrogen-3 of the ring may be a binding or affinity site on the enzyme.

We have also demonstrated that F_3TDR inhibits the nucleoside phosphorylase cleavage of FUDR to FU, which suggests that the new analog might enhance the chemotherapeutic effectiveness of FUDR[18].

We have screened F_3T (which is inactive) and F_3TDR against various transplanted tumors in mice and compared it with FUDR. Against Sarcoma-180, F_3TDR is less effective than FUDR, and this was also found wih the Ehrlich ascites carcinoma. In L-1210 leukemia the two antimetabolites have about the same activity. However, F_3TDR produces greater inhibition of the growth of Adenocarcinoma 755 with less toxicity than does FUDR, indicating that this new analog may have some clinical chemotherapeutic promise[19].

A preliminary study of the metabolic degradation in mice has shown that there is no breakdown of the pyrimidine ring, that F_3TDR is cleaved enzymatically to F_3T and that there is a further small conversion to 5-COOH-U; no other catabolites have been detected (C. Heidelberger and B. Kampschroer, unpublished data).

A summary of the metabolism and mechanisms of action of the two series of fluorinated pyrimidines is given in Fig. 18. The 5-fluorouracil series is incorporated into RNA, not DNA, and the nucleotide, FUDRP, that is produced metabolically, inhibits the enzyme, thymidylate synthetase. The

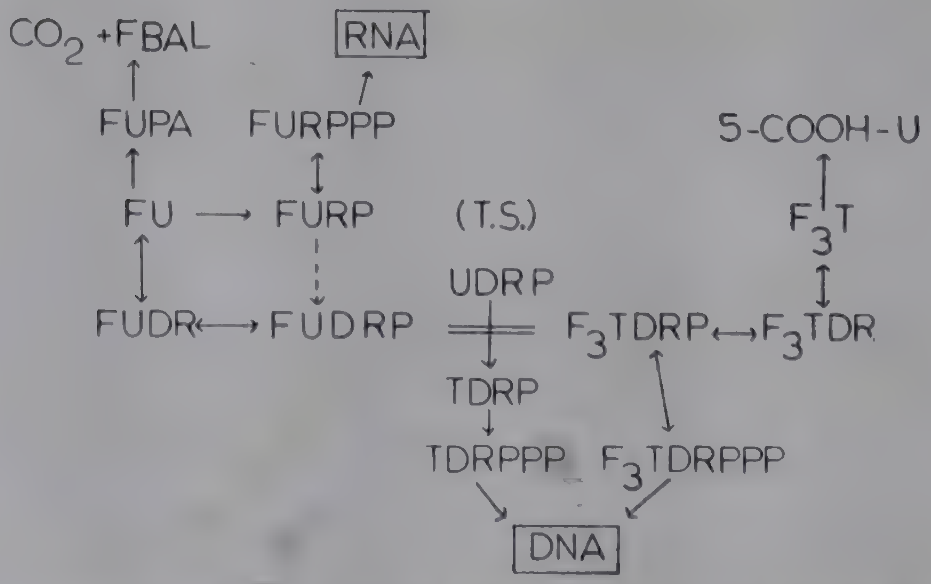


FIG. 18—SUMMARY OF METABOLISM AND BIOCHEMICAL EFFECTS OF THE TWO SERIES OF FLUORINATED PYRIMIDINES

trifluorothymine series is incorporated into DNA, and the nucleotide, F_3TDRP, inhibits the same enzyme.

It is hoped that in this short presentation it has been possible for me to outline the rationale whereby two new pyrimidine antimetabolite series were devised, one of which has shown clinical utility in the treatment of patients with far advanced cancer. A discussion of their biochemical mechanisms of action as determined primarily in our laboratory has been given.

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The Low Molecular Weight Ribonucleic Acids of *Escherichia coli* Ribosomes

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The greatest part of the RNA in rapidly growing cells is associated with basic proteins in the form of ribosomes. In *Escherichia coli* as in many other bacteria two fundamental types of ribosomes have been identified: the 30 S ribosomes and the 50 S ribosomes. Under appropriate conditions of divalent cation concentration and ionic strength, one 30 S particle unites with one 50 S particle to give one 70 S particle[1]. The 70 S ribosomes are the particles which are functional in protein biosynthesis. In the course of polypeptide bond formation, several 70 S ribosomes become linked to one molecule of messenger RNA[2–5] forming polysomes[6] or ergosomes[7]. Each 70 S particle is, therefore, supposed to have at least one binding site for messenger RNA. On the basis of experiments in which 50 S ribosomes and 30 S ribosomes have been separately mixed with artificial or natural messenger RNA, it has been proposed that the messenger RNA binding site is specifically located on the 30 S ribosome[8,9].

Another site has been characterized on 70 S ribosomes and is concerned with the binding of transfer RNA[10]. This binding may occur even in the absence of messenger RNA and peptide bond formation, provided a high concentration of Mg^{2+} be present. But when a growing peptide chain is actually linked to the transfer RNA molecule, as it happens in the course of protein biosynthesis, there is a large increase in the stability of the system with a concurrent decrease in the Mg^{2+} concentration needed to ensure attachment of transfer RNA to the ribosome[10,11]. The 50 S moiety of the 70 S ribosome has been unequivocally recognized as bearing the transfer RNA binding site[10]. All transfer RNAs, regardless of their amino acid specificity, can be bound to this site. They do not need to be charged with amino acids but they must possess an intact . . . pCpCpA terminal sequence[10,12]. It has, therefore, been suggested that the binding of transfer RNA could involve both Mg^{2+} bridges and base pairing and hydrogen bond formation between this sequence and a complementary sequence present on the 50 S ribosome[10].

As a result of these various interactions, the deproteinization, e.g. by phenol treatment, of ribosomes prepared from growing cells may be expected to liberate various types of RNA molecules, which can be fractionated by chromatography on methylated serum albumin columns[13–15].

In the case of 70 S ribosomes extracted from cells grown for several generations in the presence of ^{32}P and purified by sedimentation in a sucrose concentration gradient[16], about 95% of the extracted ^{32}P RNAs appear in two peaks, eluted by high concentrations of NaCl (Fig. 1). These two peaks correspond respectively to the 16 S RNA and the 23 S RNA, which are known to constitute the bulk of ribosomal RNA[17,18]. Low molecular weight ^{32}P RNAs, corresponding to *c.* 3% of the RNA extracted from the 70 S ribosomes in the particular experiment of Fig. 1, are eluted by lower NaCl concentrations, between fraction 25 and fraction 47 (Fig. 1), giving two distinct, but incompletely separated peaks.

It can be shown that the radioactive products present in the first peak have the same chromatographic properties as transfer RNA prepared from *Esch. coli* cytoplasm by Tissières's technique[19]. The presence of transfer RNA on *Esch. coli* 70 S ribosomes has already been demonstrated by Elson[20] and can be accounted for on the basis of the results of Cannon *et al.*[10] The

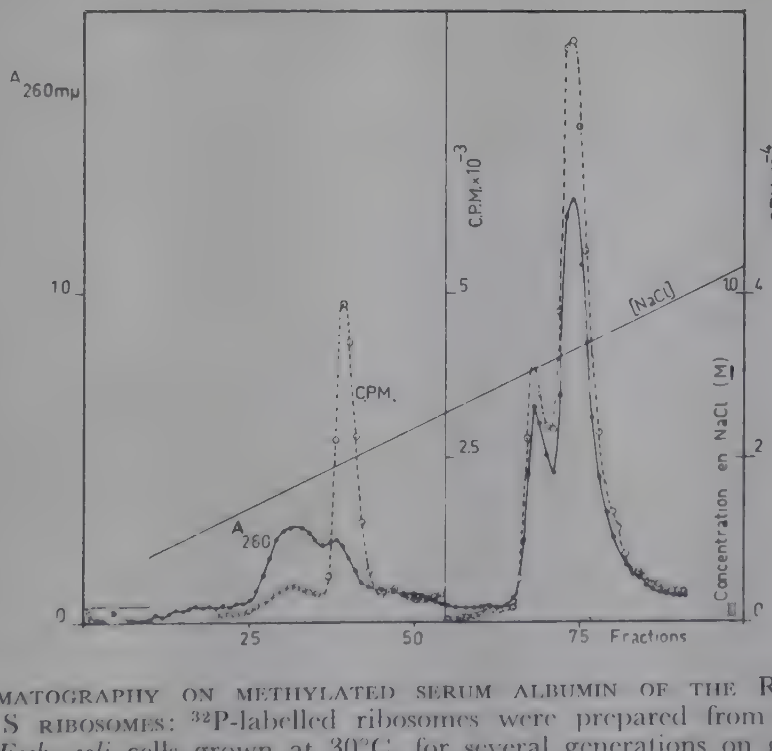

FIG. 1—CHROMATOGRAPHY ON METHYLATED SERUM ALBUMIN OF THE RNAS EXTRACTED FROM 70 S RIBOSOMES: ^{32}P-labelled ribosomes were prepared from homogenates of ML 308 *Esch. coli* cells grown at 30°C. for several generations on glucose minimal medium, containing 5 mC/*l*, of $^{32}PO_4Na_2H$. They were purified by sedimentation in a sucrose gradient (5-20%) prepared in 0·05*M* Tris-HCl buffers, *p*H 7·6, 0·01*M* $MgCl_2$. After deproteinization with phenol in the presence of 3·5 mg./ml. bentonite, unlabelled carriers were added, the RNAs were recovered by 3 alcoholic precipitations, and the mixture was chromatographed (Fraction volume=5 ml.)

amount of transfer RNA found in the analysis of Fig. 1 would correspond to less than one molecule of transfer RNA per ribosome, if we assume the molecular weights of transfer RNA, 16 S RNA and 23 S RNA to be 26,000, 550,000 and 1,100,000, respectively. In other preparations of 70 S ribosomes, especially in those which are obtained by sedimentation at 105,000 *g*, the amount of transfer RNA varies between 1 and 2 molecules per ribosome as a function of the number of washings and of the ionic strength of the medium in agreement with the results of Cannon *et al.*[10]

An analysis of the products extracted from ^{32}P-labelled 50 S ribosomes, prepared in the presence of $10^{-4}M$ Mg^{2+} by sedimentation in a sucrose concentration gradient, is shown in Fig. 2. Deproteinization of 50 S ribosomes mainly liberates 23 S RNA, but also produces a small amount of 16 S RNA. It is generally considered that the main structural RNA component of mature 50 S ribosomes actually is 32 S RNA. The presence of 16 S RNA in extracts of 50 S ribosomes has been attributed to "native" 50 S ribosomes, which are supposed to represent intermediates in the process of ribosome synthesis[21–24]. At the same time, deproteinization of 50 S ribosomes again liberates some low molecular weight RNA, eluted between fraction 25 and fraction 47 (Fig. 2). But 96% of the radioactivity recovered in these fractions is now present in the narrow peak centred at fraction 43.

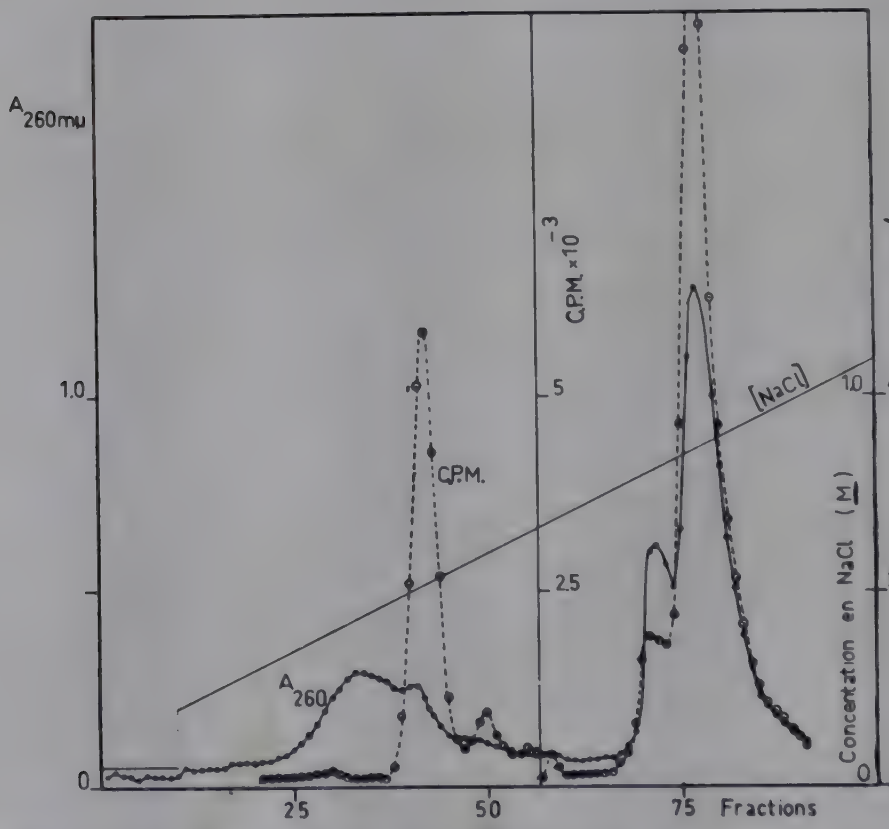

FIG. 2—CHROMATOGRAPHY ON METHYLATED SERUM ALBUMIN OF THE RNAS EXTRACTED FROM 50 S RIBOSOMES: Same conditions as under Fig. 1. 50 S ribosomes were prepared by suspending 70 S ribosomes in 0·05M Tris-HCl, *p*H 7·6, $10^{-4}M$ $MgCl_2$, dialysing for 3 hr against three changes of this buffer and sedimenting in a sucrose concentration gradient prepared in the same buffer

During exposure to low Mg^{2+} concentration, most of the transfer RNA, which was present on the original 70 S ribosomes, presumably on the 50 S moiety[10], has, therefore, been washed off. The traces of transfer RNA, which can still be detected on purified 50 S ribosomes can probably be interpreted as molecules of transfer RNA, which were linked to growing peptide chains prior to phenol deproteinization[11].

Analysis of purified 30 S ribosomes reveals the presence in these ribosomes of the 16 S component only and the complete absence of the ribosomes of low molecular weight RNAs. These results suggest that *Esch. coli* ribosomes contain a small molecular weight RNA, distinct from transfer RNA by its behaviour on methylated serum albumin columns. This RNA is linked specifically to the 50 S moiety of the 70 S ribosomes in such a way that it does not dissociate from the ribosomes upon exposure to moderate ionic strength media containing $10^{-4}M$ Mg^{2+}. In sucrose concentration gradients, this RNA sediments slightly faster than transfer RNA. For the sake of brevity, it will, therefore, be referred to in the following as 5 S RNA, in order to distinguish it from transfer (or 4 S) RNA. This does not imply that its sedimentation coefficient is exactly 5 S under any particular set of sedimentation conditions.

In most of the experiments described here the ML 308 strain of *Esch. coli* has been used. But 5 S RNA has also been detected in several K_{12} strains.

The size and/or the conformation of 5 S RNA are sufficiently different from those of transfer RNA to allow a partial separation of these two RNAs

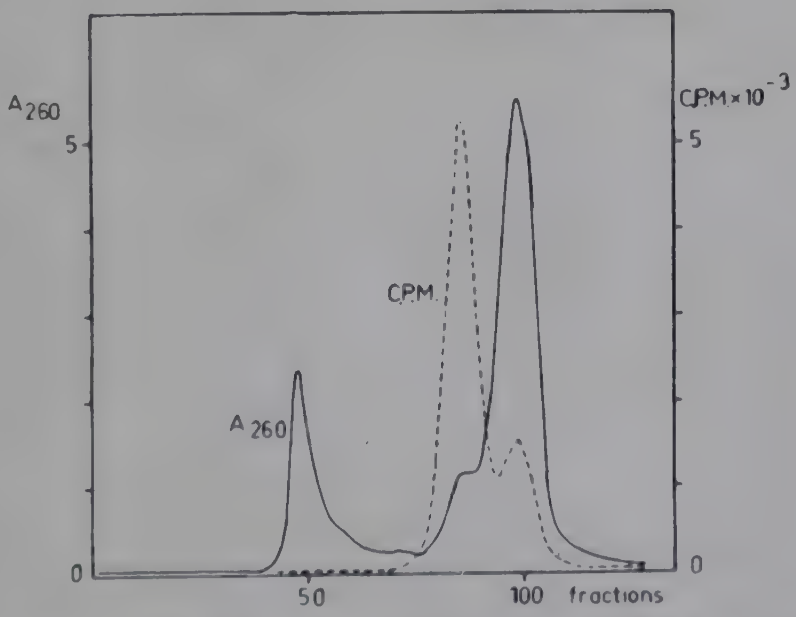

FIG. 3—CHROMATOGRAPHY ON A SEPHADEX G 200 COLUMN OF 5 S RNA: ^{32}P-RNA was recovered from the peak centred at fraction 39 in the chromatography of Fig. 1 after addition of a RNA carrier prepared by suspending unbroken *Esch. coli* cells in phenol-water. The mixture of RNAs was dissolved in $0{\cdot}05M$ sodium acetate buffer, *p*H 5·0 and filtered through a 2·5 cm. × 80 cm. Sephadex G 200 column (Fraction volume = 3 ml.)

on Sephadex G 200 columns (Fig. 3). In the experiment of Fig. 3, ^{32}P-labelled 5 S RNA extracted from 70 S ribosome and partially purified by methylated serum albumin chromatography has been mixed with carrier RNA obtained by direct treatment of whole *Esch. coli* cells with phenol. This type of carrier contains transfer RNA which is responsible for the main peak of optical density in Fig. 3. The peak shows on its heavy side a shoulder, which apparently corresponds to 5 S RNA. A small amount of high molecular weight ribosomal RNA, which comes through the column without retardation, was also present in the carrier.

5 S RNA behaves as a metabolically stable product, since its amount in 50 S ribosomes is not diminished when these ribosomes are prepared from ^{32}P-labelled cells either after chasing for one generation with an excess of ^{32}P or after incubating for 30 min. in the presence of $5{\cdot}10^{-3}M$ dinitrophenol[25].

5 S RNA has been isolated from ribosomes and methylated serum albumin chromatography of RNAs extracted from the cytoplasmic fraction of *Esch. coli* cells does not give any clear indication of the presence of 5 S RNA in cytoplasm. Moreover, the results of analyses of total RNA samples extracted from *Esch. coli* ML 308 grown on various media may be taken as evidence for the ribosomal nature of 5 S RNA. Under our experimental conditions, when the growth rates were higher than 0·40, we found a constant ratio between the amount of transfer RNA and the amount of 16 S RNA and 23 S RNA. This ratio corresponded to about 10 molecules of transfer RNA per ribosome.

But in cells grown on media in which the growth rate was limited by the nature of the nitrogen source and maintained below 0·25, the number of transfer RNA molecules per ribosome was no longer constant and increased when the growth rate decreased[26]. Under these circumstances, it was found that the 5 S RNA content changed in the same way as the ribosome content.

The nucleotide composition of 5 S RNA is reported in Table 1. 5 S RNA is more similar to transfer RNA than to 16 S and 23 S RNAs in its overall composition. Nevertheless it is noticeable that the preparation used in these analyses did not liberate more 2′, 3′-Ψ UMP or (2′, 3′), 5′-GDP than could be expected on the basis of the amount of transfer RNA present as a contaminant. No exact estimation of the methylated base nucleotides has been made, but the amount of these products was also very low in our 5 S RNA samples. Therefore, 5 S RNA does not probably contain any of the odd bases, which are characteristic of transfer RNA. Moreover, alkaline degradation of 5 S RNA liberates a nucleoside diphosphate, which has been identified as (2′, 3′), 5′-UDP. Since no other nucleoside diphosphates have been detected in significant amounts, we may assume that all the molecules of 5 S RNA are terminated at their 5′ phosphate end by 5′-UMP. The amount of (2′, 3′), 5′-UDP found in the hydrolysates

TABLE 1—NUCLEOTIDE COMPOSITION OF ALKALINE HYDROLYZATES OF *Esch. coli* RNAs

	% of recovered nucleotides			
	Transfer	5 S	16 S	23 S
AMP	18·4	18·5	24·3	24·6
CMP	28·4	28·9	21·8	21·4
GMP	30·8	34·8	32·0	31·8
UMP	18·8	16·6	21·8	22·2
ΨUMP	2·4	0·18	not detected	not detected
GDP	1·18	0·08	..	..
UDP	0·17	0·90	..	..

^{32}P-labelled RNAs, purified by methylated serum albumin chromatography, were hydrolysed in the presence of yeast sRNA as a carrier (0·3 *N* KOH, 37°C., 18 hr) and the hydrolysates, after neutralization with Dowex 50-H$^+$ resin, were analysed by chromatography on Dowex 1-formate.

would then correspond to a chain length of 105±5 nucleotides and to a molecular weight of 35 900±1 700.

With a knowledge of an approximate value of 5 S RNA molecular weight, it became possible to calculate the number of 5 S RNA molecules present in various ribosome preparations. In 70 S ribosomes, the amount of 5 S RNA found would correspond to 1·09 molecules per ribosome (average value of 5 determinations—extreme values: 0·95-1·22 molecules per ribosome) and in 50 S ribosomes, to 0·93 molecule per ribosome (average value of 5 determinations—extreme values: 0·85-1·00). It is specially important to notice that the amount of 5 S RNA in 70 S ribosomes did not depend on the method used for their preparation, while, as has been pointed out before, the amount of transfer RNA found on 70 S ribosomes was very much dependent on the experimental conditions.

^{32}P-5S RNA samples were exposed to snake venom phosphodiesterase[28–31] and the rate of liberation of the nucleotides was followed (Fig. 4). 5′-UMP was the only nucleotide liberated in significant amount under the conditions used. On the basis of the molecular weight previously determined, the amount of 5′-UMP released after prolonged exposure to the enzyme would correspond to 0·79 mole per mole of 5 S RNA. It is very likely, therefore, that 5 S RNA is made of polynucleotide chains about 100 nucleotide long and bearing at both ends a residue of uridylic acid.

Transfer RNA was hydrolysed with snake venom phosphodiesterase under conditions identical with those used in the experiment of Fig. 4. The rate of liberation of both 5′-AMP and 5′-CMP from the . . . pCpCpA sequence was much more rapid than the rate of liberation of 5′-UMP

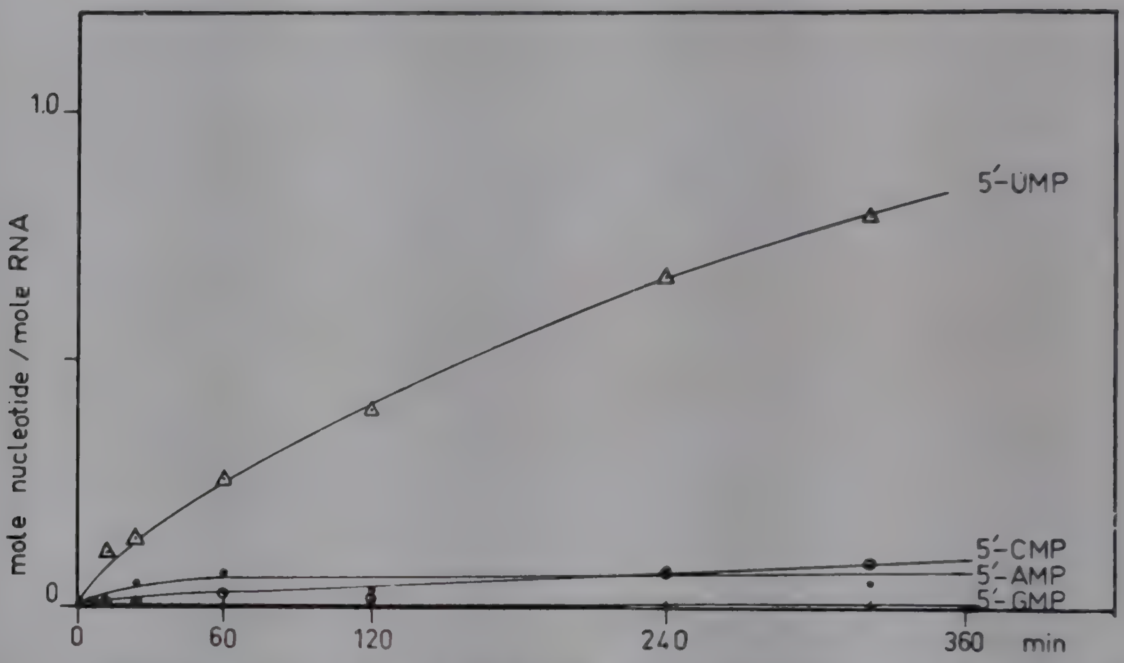

Fig. 4—Degradation of 5 S RNA with snake venom diesterase: ^{32}P-5 S RNA (1 mg.) was hydrolysed with 10 mg. of snake venom phosphodiesterase (Worthington) in 0·12 ml. of 0·05*M* Tris-acetate buffer, *p*H 8·8, 0·05*M* magnesium acetate at 14°C. Mononucleotides were separated from undegraded RNA by prolonged paper chromatography (ammonium isobutyrate, *p*H 3·7) and the separation of 5′-UMP from 5′-GMP was then achieved by electrophoresis in 0·02*M* sodium citrate, *p*H 3·5

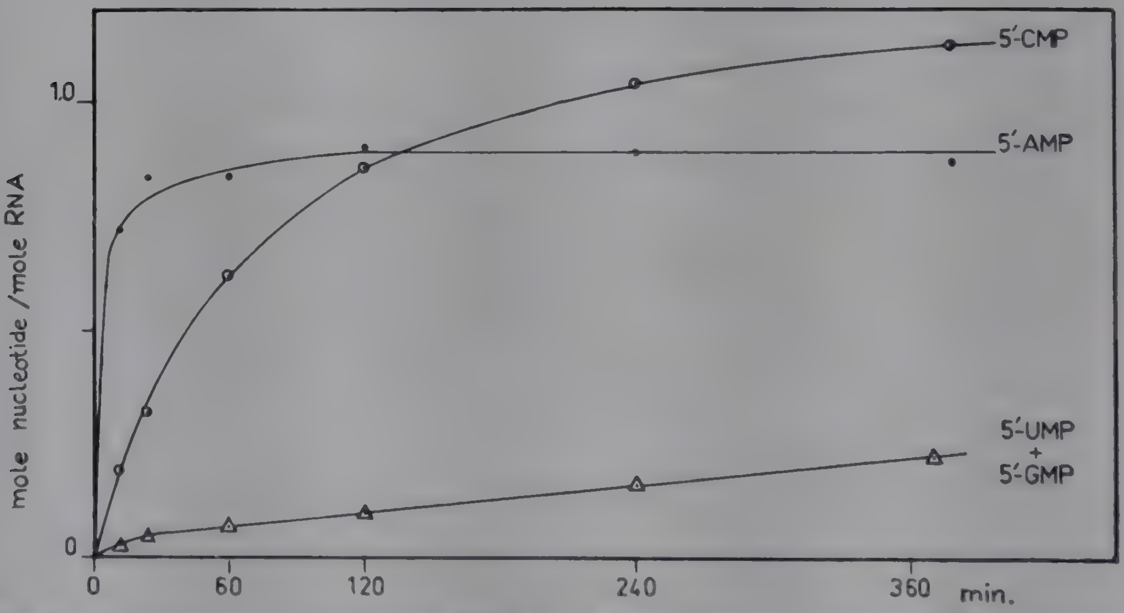

Fig. 5—Degradation of transfer RNA with snake venom diesterase: Same conditions as under Fig. 4

from 5 S RNA (Fig. 5). Since we do not know with any certainty what is the influence of the nature of the base on the rate of hydrolysis, this difference cannot be unequivocally interpreted, but it does suggest that transfer RNA and 5 S RNA do not possess identical conformations in solution.

Through all these evidences, 5 S RNA appears as a well defined product, chemically very different from transfer RNA. The results of Table 2 show that it is also functionally different. The samples of low molecular weight RNAs used in these experiments were mixtures of transfer RNA and 2 S RNA, obtained from 70 S ribosomes or 50 S ribosomes prepared by sedimentation at 105,000 *g*. The acceptor activities of these preparations were almost exactly those which could be predicted on the basis of the transfer RNA content as determined by chromatographic analysis. Previous treatment of the RNAs at *p*H 10 and 37°C. for 50 min. did not affect the activity. 5 S RNA is, therefore, unable to bind amino acids under conditions where transfer RNA functions as an acceptor.

5 S RNA is firmly linked to the 50 S ribosomes, in media of moderate ionic strength containing $10^{-4}M$ Mg^{2+}. But it can actually be liberated from the 50 S particles without a complete destruction of their structure. This happens, for example, when 50 S ribosomes are treated with high concentrations of alkaline ions, Li^+ being more efficient than Na^+. At concentrations of LiCl higher than $2M$ and in the presence of $10^{-3}\,M$ $MgCl_2$ and of $5—10^{-2}M$ Tris-HCl, *p*H 7·6, 50 S ribosomes lose all of their 5 S RNA. They also lose part of their proteins and their sedimentation coefficient in a sucrose concentration gradient is reduced.

Nevertheless *Esch. coli* ribosomes appear to be more resistant to LiCl

TABLE 2—ACCEPTOR ACTIVITY OF LOW MOLECULAR WEIGHT RNAs EXTRACTED FROM *Esch. coli* RIBOSOMES

Exp.	RNA	Amino acids incorporated (c.p.m./mg. RNA)	Activity (% of control)
I	transfer RNA control	21,960	..
	RNA from 70 S ribosomes	9,750	44
II	transfer RNA control 1	19,400	..
	transfer RNA control 2	20,400	..
	RNA from 50 S ribosomes	2,450	12

Transfer RNA was prepared by the method of Tissières[19]. RNAs from ribosomes were prepared by phenol deproteinization of particles washed twice by sedimentation at 105,000 *g*. High molecular weight RNAs were eliminated by 3 precipitations at 0°C. with $1M$ NaCl followed by chromatography on DEAE-cellulose. 70 S ribosomes were prepared in $0{\cdot}05M$ Tris-HCl buffer, *p*H 7·6, $0{\cdot}01M$ $MgCl_2$. 50 S ribosomes were prepared in $0{\cdot}05M$ Tris-HCl buffer, *p*H 7·6, 10 M $MgCl_2$. The RNA samples used in Exp. I were incubated at *p*H 10·0 and 37°C. for 50 min. before measuring the activity.

Acceptor activity was measured with ^{14}C-amino acids from an algal protein hydrolysate by conventional techniques in the presence of *Esch. coli* cytoplasmic fraction dialysed for 15 hr against $0{\cdot}05M$ Tris-HCl, *p*H 7·6, $5\text{-}10^{-3}M$ magnesium acetate, and $10^{-3}M$ reduced glutathione.

than reticulocyte ribosomes, the proteins of which are completely dissociated from RNA in $2M$ lithium chloride[33].

Nothing definite is known about the exact role of 16 S and 23 S RNAs in protein biosynthesis. Their association with some of the ribosomal proteins appears to be very intimate and it is possible that their only function is to furnish the structural basis on which the synthesizing machinery is built up. The presence of a small molecule of RNA in the 50 S ribosomes, which can be dissociated from the main 23 S RNA constituent, without the complete destruction of the ribonucleoprotein particle, has now been established. This small RNA molecule could perhaps play a more active part in protein biosynthesis than the 16 S and 23 S RNAs and experiments are now in progress to study the function of 5 S RNA.

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Analysis of Base Composition in Chromosomes of Higher Plants Through Response to Mutagens

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Several chemicals are known to attack the four common nitrogenous bases of desoxyribonucleic acid (DNA) and utilizing the forward and reverse mutations induced by such chemicals, a model of molecular mutagenesis has been proposed in bacteriophages by Freese[1]. In higher organisms, however, such a precise study with these mutagens is difficult, in view of the complex nature of the genetic and chromosomal organization. Thus chemicals whose mutagenic actions can be explained on the basis of base transitions (e.g. base analogues), transversions (e.g. alkylating agents) and duplications and deletions (e.g. acridine derivatives) may not be as specific as they are in microorganisms. In the present paper, an attempt is made to summarize our results on mutagenic action of several of these chemicals on higher plants. The data are presented in two parts, the first part dealing with studies on chromosome breakage in root meristems of a few species of plants and the second part dealing with mutation studies in barley. The results are interpreted on the assumption that the observed effects arise from the action of these chemicals on DNA of the chromosomes.

MATERIALS AND METHODS

The mutagens which have been employed for cytological studies, the mode of treatment with them, and the bases which are affected, are tabulated in Table 1. The biological materials chosen are of the type in which chromosomal changes could be studied with considerable precision. The list of plant material used and their chromosome numbers are tabulated below:

(1)	*Vicia faba*	2n=12
(2)	*Ornithogalum* sp.	2n= 6

(3) *Allium cepa* $2n=16$
(4) *Hordeum vulgare* $2n=14$

A detailed study of the localization of breaks was done in *V. faba* and *Ornithogalum*, while the other two species have mainly been used to study the effect of these mutagens on centromeric or the secondary constriction regions of different chromosomes.

RESULTS

Studies in *Vicia faba*

Since the first report by Ford[2] that nitrogen mustard breaks chromosomes of *V. faba* in a non-random manner, several chemical mutagens have been tested and found to have localized effect in this material, e.g. maleic hydrazide, 8-ethoxy caffeine and myleran have been shown to induce chromosome breaks preferentially at different points of chromosomes. We have studied the action of ethyl methanesulfonate (EMS), hydroxylamine (HA) and 5-fluorodeoxyuridine (5-FUDR), on the chromosomes of *V. faba*. Sprouted roots were treated and scored for different revival times[3].

In the chromosome complement of *V. faba*, 4 distinct types of chromosomes can be distinguished from one another (Fig. 1), i.e. a pair of median constricted long, satellited chromosomes (M chromosome), 5 pairs of short chromosomes of which one pair has relatively longer short arm with an extra constriction in the short arm (Sa), one pair which is almost telocentric, with negligible size of short arm (Sc), and 3 other pairs which have intermediate size of short arms, not distinguishable from each other (Sb). The chromosomes of *V. faba* are known to possess heterochromatic regions, which show up after cold treatment[4]. The immediate effect of the treatment with hydroxylamine or ethyl methanesulfonate was the lighter Feulgen stainability of some of those heterochromatic regions which appeared as extra constriction in the chromosome, occupying a larger diffused area than the constriction (Fig. 2). These are not visible either in control or 5-FUDR treated material. The breaks realized following

TABLE 1—MECHANISM OF ACTION OF CHEMICAL MUTAGENS

Material	Form in which used	Base affected
Hydroxylamine	High salt concentration, *p*H 7·0	Cytosine
Ethylmethane sulfonate	Aqueous solution, *p*H 7·0	Guanine
Myleran	Aqueous solution, *p*H 7·0	Guanine
5-Fluorodeoxy-uridine	Aqueous solution, *p*H 7·0	Thymine
Methylethane sulfonate	Aqueous solution, *p*H 7·0	Guanine
5-Aminouracil	Aqueous solution, *p*H 7·0	Thymine

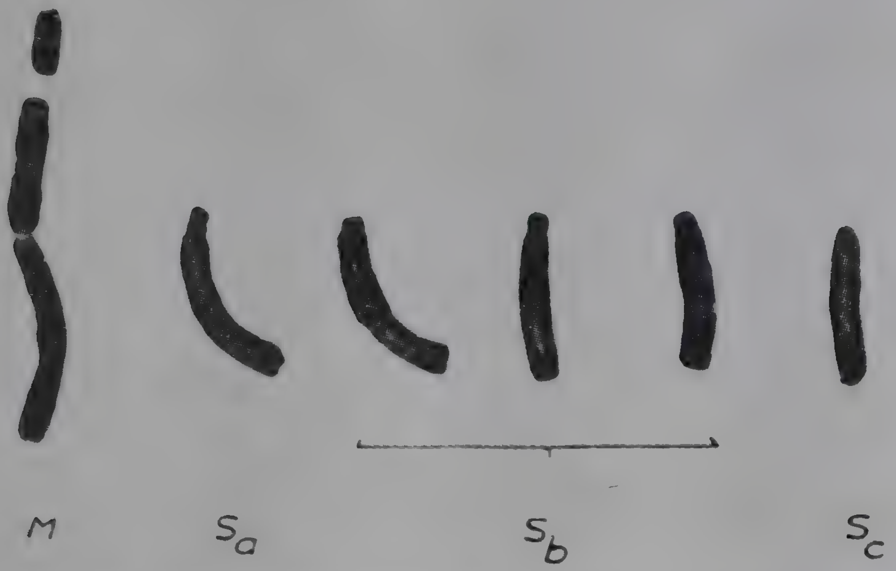

Fig. 1—Chromosome complement of *Vicia faba* (n=6) and the grouping according to their identifiability

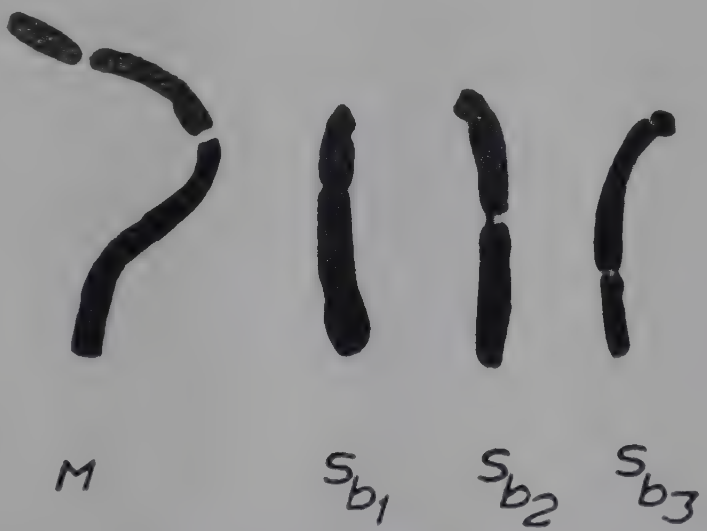

Fig. 2—The localization of Feulgen-negative heterochromatic segments visible in the first cell cycle, following EMS or HA treatment

HA and EMS, were exclusively confined to these points. HA (1 mg./ml.) induced a larger number of breaks than EMS at the dosages tried. Doses higher than 0·3% of EMS for 2 hr and 1 mg./ml. of HA for 2 hr were lethal. Only 5 points, i.e. M_1, M_2, Sb_1, Sb_2 and Sb_3, were involved in breakage, Sa and Sc being completely devoid of breaks in the arms. The initiation and fate of breaks at these points have been studied critically.

Another breakage point, which was common in all the six pairs of chromosomes, was the centromeric region. The centromere broke frequently leading to centric fragments and telocentrics at later cell generations. Loss of centromeric activity leading to the exclusion of one or more chromosomes was observed in later cell cycles.

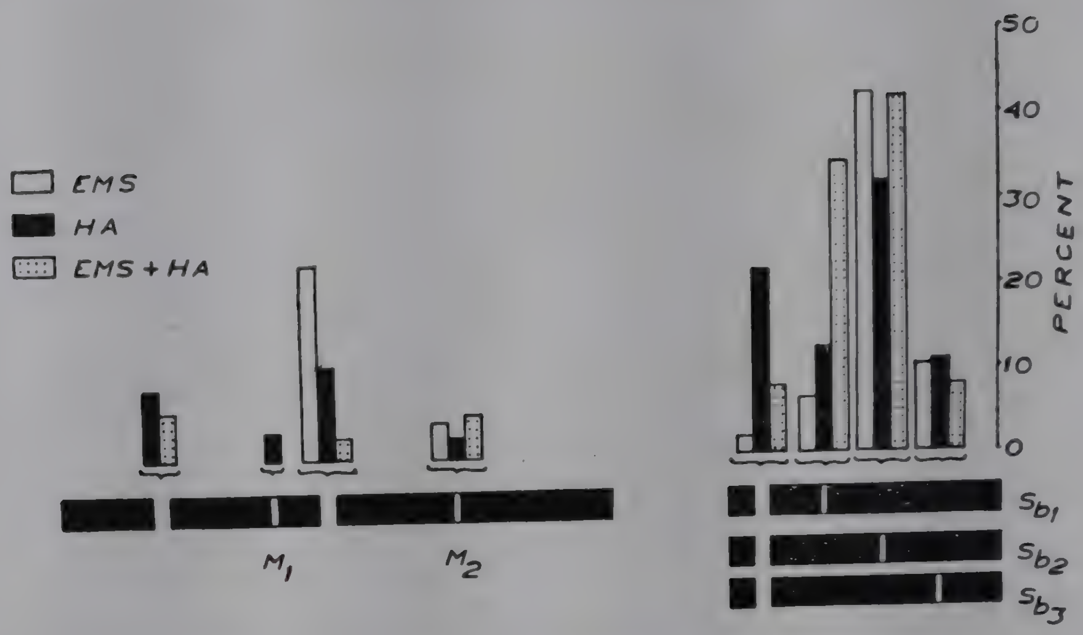

FIG. 3—RELATIVE FREQUENCY OF BREAKS AT VARIOUS BREAKAGE POINTS IN *Vicia faba* FOLLOWING EMS AND HA TREATMENT

The relative frequency of breaks at different breakage points is diagrammatically represented in Fig. 3.

With 5-FUDR treatment, there was a general extreme breakage, spread all over the chromosomes, though high localization could be seen in the sat-region and in the heterochromatic region close to the centromere, in the short arm of the long chromosomes. Centromeric breaks were not recorded with this treatment, nor were breaks obtained at any of the specific breakage points known to be susceptible to HA or EMS.

Studies in ***Ornithogalum***

This species has 3 pairs of chromosomes, each of which is identifiable from the other, though all the 3 pairs are subterminal in their centromeric position. There are two long pairs, of which one is satellited; this appears as lightly stained whiskers at the terminal end, and is distinguishable from the other long pair. The third pair is smaller (Fig. 4). Myleran (40 mg./ml./hr), methyl ethanesulfonate (MES) (0·06% aqueous solution at *p*H 7·0 for 30 min. at 30°C.), and hydroxylamine (1 mg./ml./hr) were used for treatments. There were 9 breakage points identifiable, 3 points per chromosome, common to all the chemicals tested. As in *Vicia faba*, the breakage points appeared as lightly Feulgen-stained spots, in the first fixation, which in later fixations turned into chromatid or chromosome breaks. In severely affected cells, these regions appeared to be completely devoid of any stainable material with only a small Feulgen-positive thread connecting them. The breakage points for these chemicals were found to overlap. Subchromatid exchanges as well as chromatid breaks were realized at later revival times in some of the cells at these points, indicating the induction of subchromatid breakage, which remained latent and showed up at later

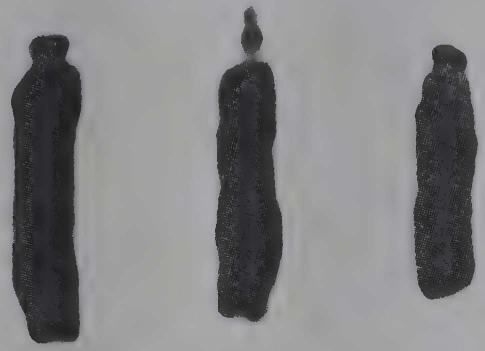

FIG. 4—CHROMOSOME COMPLEMENT OF *Ornithogalum* (n=3); ALL THE THREE CHROMOSOMES IDENTIFIABLE

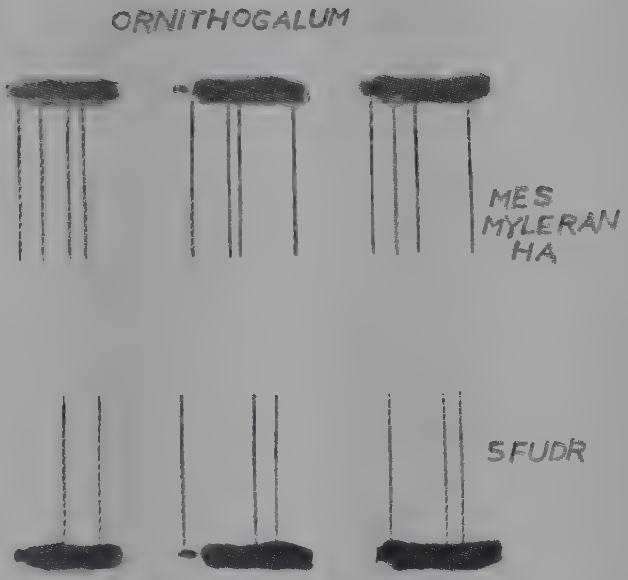

FIG. 5—CHROMOSOME BREAKAGE POINTS IN *Ornithogalum*, FOLLOWING MES, HA, MYLERAN AND 5-FUDR TREATMENT

fixations. The various points of breakage by these 3 agents are depicted in Fig. 5. Breaks at the centromeric regions were also common, and were of particularly high frequency in MES-treated material. The breakage points induced by 5-FUDR do not overlap with most of the points of attack by HA, MES or myleran (Fig. 5).

Studies with *Allium cepa* and *Hordeum vulgare*

Since the chromosomes are not identifiable, only breaks at the centromere and secondary constrictions were taken into account in the case of *Hordeum* and only centric breaks were studied in the case of *Allium*. The mutagens used were EMS, HA, MES, 5-fluorodeoxyuridine, myleran and 5-aminouracil. With the alkylating agents as well as HA, a very high frequency of centromeric breaks occurred in all the 7 pairs of chromosomes of barley, many times resulting in homologous and non-homologous centric exchanges (Fig. 6). 5-Fluorodeoxyuridine induced preferential breakage at the satellite region, with a very low frequency of breaks at the centromere. In the case

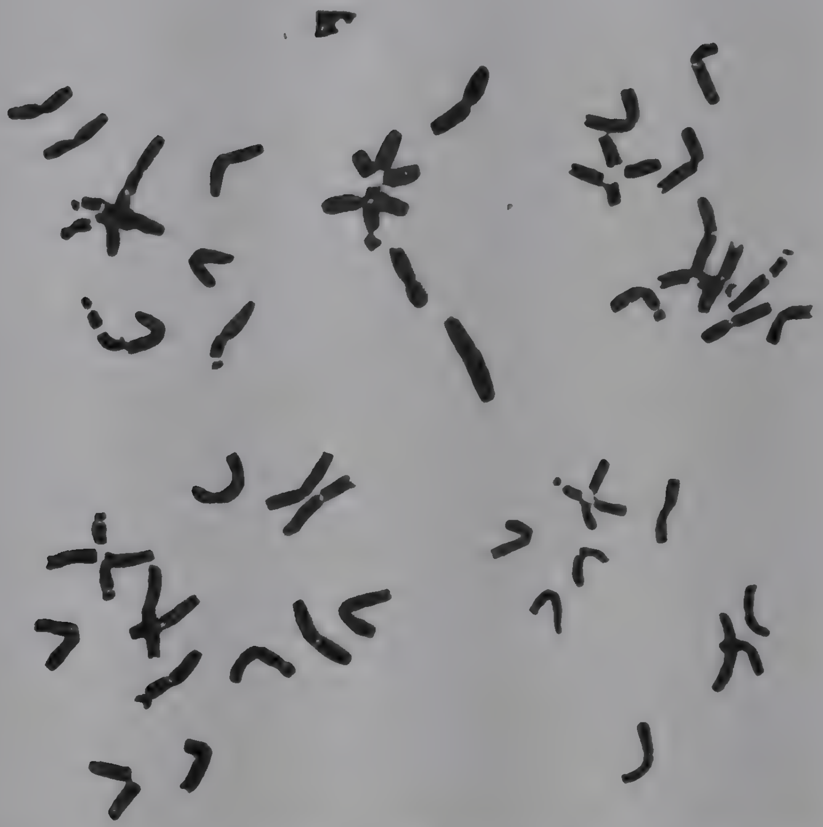

FIG. 6—SOMATIC PLATES OF *Hordeum vulgare* SHOWING CHROMOSOME EXCHANGES INVOLVING CENTROMERES, FOLLOWING EMS TREATMENT

of *Allium*, there are centromeric breaks induced by myleran, as well as 5-aminouracil, though the chromosomes involved do not seem to overlap.

DISCUSSION

Mechanism of action of chemical mutagens

Alkylating agents. The various possible mechanisms, by which the alkylating agents can act on DNA, have been summarized by Freese[1]. The major effect is expected to be on the phosphate groups of the DNA, and then on the purine bases, especially guanine. Since, (*a*) there is no method of differentiating the chromosome breakage caused by either of the above-mentioned reactions, (*b*) the phosphate groups are present all over the DNA molecule (and hence the chromosomes) and an effect on phosphate groups should lead to random chromosome breakage, and (*c*) there is a very high localization of breaks, the action on the guanine moiety has been assumed to be important in chromosome breakage by alkylating agents. An ethylated base can induce mistakes on further replication, or can separate from the DNA leaving it depurinated. Such changes in chromosomes can cause coiling, condensation and duplication difficulties.

Hydroxylamine. It is known to attack pyrimidine bases, especially cytosine and uracil; at neutral *p*H, the reaction with cytosine is 30-fold over uracil. Under our experimental conditions (*p*H 7·0), we assume that the major effect of hydroxylamine is on the cytosine moiety of the chromosomes.

5-Fluorodeoxyuridine and 5-aminouracil. These two analogues are known to interfere with thymine synthesis (inhibition of thymidylate synthetase[5,6]. Thus, in these experiments, the localized effect of these agents is assumed to be due to their action on thymine.

Localization of breaks

From the study of *V. faba* and *Ornithogalum*, it is observed that the breakages induced by the alkylating agents and hydroxylamine are very specific and localized, indicating that by either of the treatments, the same regions are affected. It can be assumed that these regions are rich in guanine-cytosine pairs in DNA. These regions are not affected by agents which affect thymine synthesis. Somers and Hsu[7] have shown that hydroxylamine and 5-bromodeoxyuridine (5-BURD) induce localized breaks in mammalian chromosomes and the breakage points of these two agents do not overlap. They have interpreted their observations as due to the relative richness of these regions in GC or AT bases. Earlier studies on *V. faba* with other alkylating agents have indicated such localized effect[2,8]. The assumption that the localization of breaks is due to the G-C richness of these heterochromatic regions, finds support from the overlapping data of hydroxylamine treatment. It is evident that the heterochromatic nature of the segments (i.e. relatively less coiling) increases the susceptibility to these agents; but it is not that all the heterochromatic segments are susceptible to the agents which attack guanine or cytosine. For instance, the heterochromatic segment which is very close to the centromere on the short arm of the M chromosome (Fig. 7), was not found to break with these agents. This region, on the contrary, is preferentially broken by maleic hydrazide[4] as well as 5-FUDR[9] (also present data). Since these agents interfere with thymine synthesis, the break specific at this region may be due to its richness in

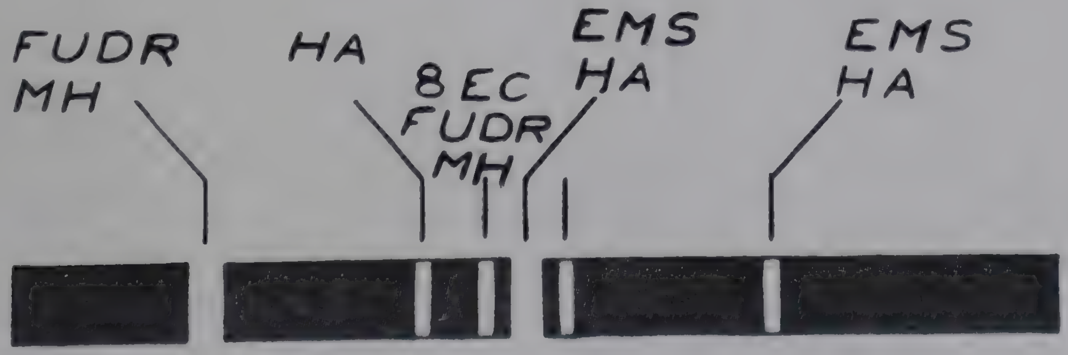

Fig. 7—Different breakage points in the M-chromosome of *Vicia faba*, following treatment with agents which specifically attack guanine, cytosine and thymine (*see* text), indicating relative richness of these regions for GC or A-T pairs of bases

adeninethymine pairs of bases. Based on the response to the specific mutagens, the relative base composition of different regions can be plotted, and such an attempt is depicted for M-chromosome of *V. faba* (Fig. 7).

The mechanism of action of these agents leading to chromosome breakage is still speculative. In the complex structure of the chromosome composed of DNA and protein, it is difficult to visualise breakage of chromosome as the consequence of alteration or replacement of a few base pairs. But in regions which are rich in either GC or AT pairs of bases, treatment with agents like 5-BUDR or EMS will alter the bases as well as the chemical nature of these bases and this might weaken the chromosome as such and introduce coiling and duplication difficulties leading to chromosome breakage.

Composition of centromeres. Centromeres are the key structure in chromosome movement during cell division. Little information is available on the chemical composition of the centromeres. In favourable preparations, this has been found to be a quadripartite structure, composed of Feulgen positive chromomeres[10,11]. In our experiments on the chromosomes of *Vicia*, *Ornithogalum* and *Hordeum*, we have observed preferential breakage at the centromere, both by alkylating agents and hydroxylamine. Earlier studies[8,12–14] have shown a similar specific centromeric breakage by alkylating agents. The high susceptibility of centromere to these agents indicates a GC rich composition of this region in these materials. A very similar situation has been reported in mammalian chromosomes by Somers and Hsu[7], where an extreme localization of breaks at the centromere following treatment with hydroxylamine was observed. These authors have also suggested possible GC composition of centromeres. This, however, does not appear to be universal, and may vary from species to species and in some cases between chromosomes of the same species. In *Allium*, it appears that some of the chromosomes have GC type of centromeres while others are of AT type, because centromeric breaks are realized following treatments which affect guanine or cytosine as well as thymine. However, this will not rule out the possibility that certain centromeres might contain all the four bases which would make them susceptible to either type of agents. Precise information with regard to the composition of the centromeres of various chromosomes in a species, throws out the possibility of inducing specific breaks at these regions and create iso- or pseudo-isochromosomes. Such stocks will be of immense help in gene mapping and linkage analysis in higher organisms.

Composition of the secondary constriction. The secondary constriction or the satellited regions are associated with nucleolar organizing activity of the nucleus. Very little is known about the structure and chemical nature of these regions. So far, two types of Feulgen-positive structures have been recognized in the sat-region of plant chromosomes, i.e. a thread-like structure[15], and a bipartite, chromomeric structure[16]. In *Vicia faba*, maleic hydrazide (MH)[4], 8-ethoxycaffeine[17] (8-EOC) and 5-FUDR[5] (also present study), have been shown to break the secondary constriction region preferentially. MH has been shown to accumulate at the nucleolar region[18] while the other agents interfere with thymine synthesis. 5-FUDR has been found to break the sat-regions in barley (present study), and 8-EOC has been shown by Moutschen-Dahmen[8] to affect these regions in barley. In *Ornithogalum*, 5-FUDR treatment completely dissolves the whisker like protuberance which is the sat-region. Thus, in these two species, the DNA of sat-region is comparatively rich in AT pairs of bases, in contrast to the GC rich centromeres of these chromosomes. Hydroxylamine at *p*H 9 breaks the sat-region specifically. At this *p*H, HA is very specific to uracil, and since this region is the site of active RNA synthesis the breakage at this region is attributable to the effect on uracil.

Mutation studies. Since the laws of genetics apply with equal validity to microorganisms and higher organisms, it will be of interest to compare the effectiveness of these agents which induce molecular mutations on higher organisms. We have extensive data on barley, and they are mentioned in brief here.

Alkylating agents. A series of mono-functional alkylating agents have been tested in barley. These chemicals are: ethyl methanesulfonate (EMS), ethyl ethanesulfonate (EES), butyl methanesulfonate (BMS), iso-butyl methane sulfonate (iso-BMS), propyl methanesulfonate (PMS), iso-propyl-methanesulfonate (iso-PMS), methyl methanesulfonate (MMS), and methyl ethanesulfonate (MES). Mutation rates have been measured both for lethal mutations (comprising various chlorophyll deficient mutants) and viable mutations (comprising those with changed characteristics and growing up to maturity). The relative efficiency of these alkyl radicals in the production of mutations is presented in Fig. 8. Ethylation was found to produce maximum mutation frequency with relatively low lethal effects (like chromosome breakage and gross exchanges). In contrast, methylation was very lethal and induced very low mutation rate. Methylating sulfonates were found to be very reactive, and induced complete killing at doses 10 times lower than ethylating agents. The relative efficiency of ethylation and methylation in higher organisms, follows the same pattern as that in micro-organisms[19]. Both propylation and butylation were found to be less potent than ethylation, but more efficient than methylation.

Indirect evidence that the primary effect of these agents is on DNA has been obtained. When the resting seeds of barley are initiated for germination under constant temperature by soaking in water with constant agitation, one can follow the initiation of the cell division in the meristomatic cells. The initials of the primordial cells, which later give rise to the root, shoot and flower primordia, start their chromosome duplication during this initial germination process. This process has been followed by utilizing tritiated thymidine as precursor. The autoradiographic studies (Natarajan

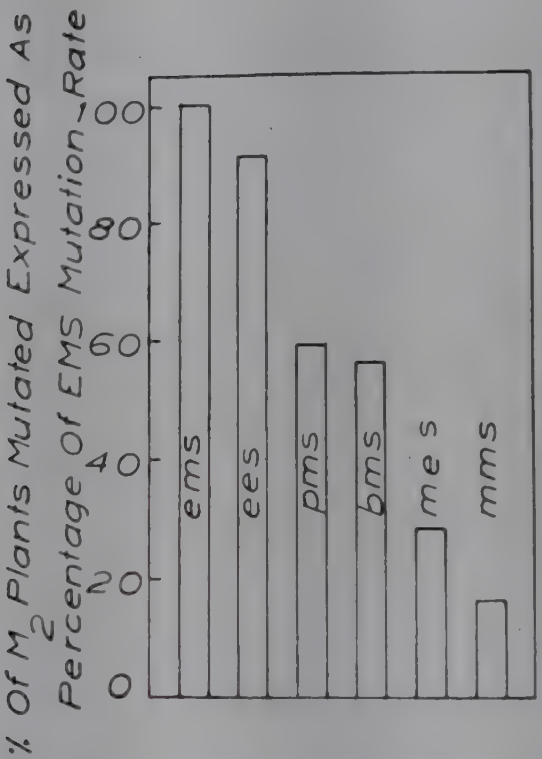

FIG. 8—RELATIVE MUTAGENIC EFFICIENCY OF DIFFERENT ALKYLATING AGENTS IN BARLEY (EMS—ETHYL METHANESULFONATE, EES—ETHYL ETHANESULFONATE, PMS—PROPYL METHANESULFONATE, BMS—BUTYL METHANESULFONATE, MES—METHYL ETHANESULFONATE, MMS—METHYL METHANESULFONATE)

and Ehrenberg, unpublished) have revealed that fresh DNA synthesis starts between 16 and 20 hr after initiation of soaking at 20°C. Experiments conducted with short duration treatments (4 hr) with ethyl methanesulfonate on seeds presoaked for different durations (0 to 28 hr), indicate a deep rise in the mutation rate at 16 to 20 hr soaking period (Fig. 9), indicating that this period is very sensitive to the mutagen used.

Base analogues

5-Bromouracil (5BU), 2-aminopurine (2AP) and 1-methyl-3-nitro-1-nitroso-8-guanidine (NNG) have been tried for mutations in barley. All of them have been found to induce extreme chromsome breakage; 2AP and 5BU have been found to radio-sensitize the material as in microorganisms. However, of these, 5-bromouracil has not yielded any mutations by itself; 2AP and NNG have induced mutation frequency to the extent of 4·0% (2AP) and 12·5% (NNG).

Photodynamic dyes

Acridine orange and methylene blue have been tried alone and in combination with radiation, for their mutagenic and radio-sensitizing properties, in barley. Both these dyes are known to have affinity for the DNA molecule[20,21]. These dyes do not induce chromosome aberration by

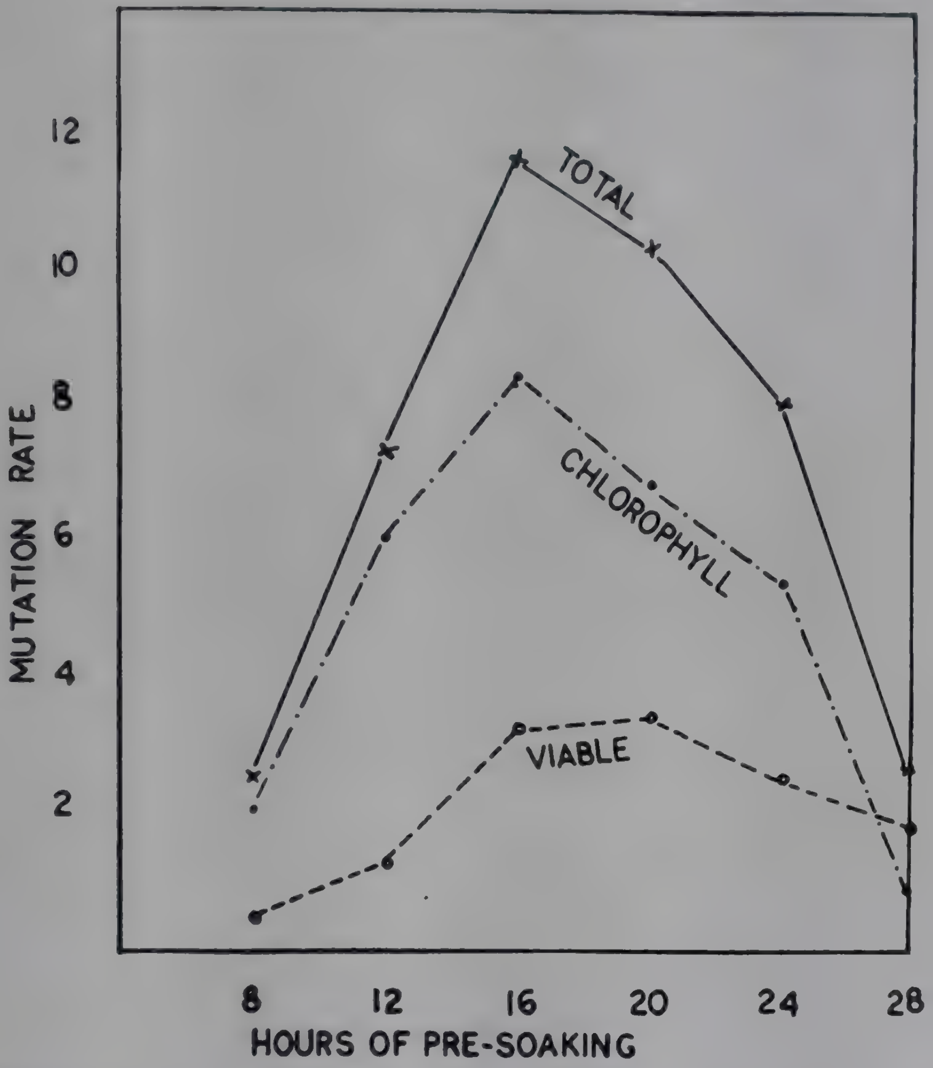

FIG. 9—DIFFERENTIAL MUTAGENIC RESPONSE OF BARLEY TO 0·6% OF EMS FOR 4 HOURS, FOLLOWING DIFFERENT DURATIONS OF PRE-SOAKING IN WATER: The peak coincides with the first replication of DNA in the cell

themselves in barley (without photo-irradiation and oxygenation). However, when combined with X-irradiation, they increase the chromosome breakage frequency by 30%. Both the dyes do not induce any mutations in barley. While acridine orange does not increase the X-ray-induced mutation frequency, methylene blue increases the mutation rate by 5 to 6-fold (Fig. 10). The differential response is perhaps due to the non-specificity of acridine orange (since acridine orange forms a complex with the whole DNA molecule) and greater specificity of methylene blue towards guanine of the DNA molecule.

Directed mutagenesis

Directed control of induced mutations is an important goal, both from fundamental and applied points of view. Experiments with microorganisms (fungi, bacteria and bacteriophage) have provided evidence for such a process of directed mutagenesis. The complicated multi-stranded structure

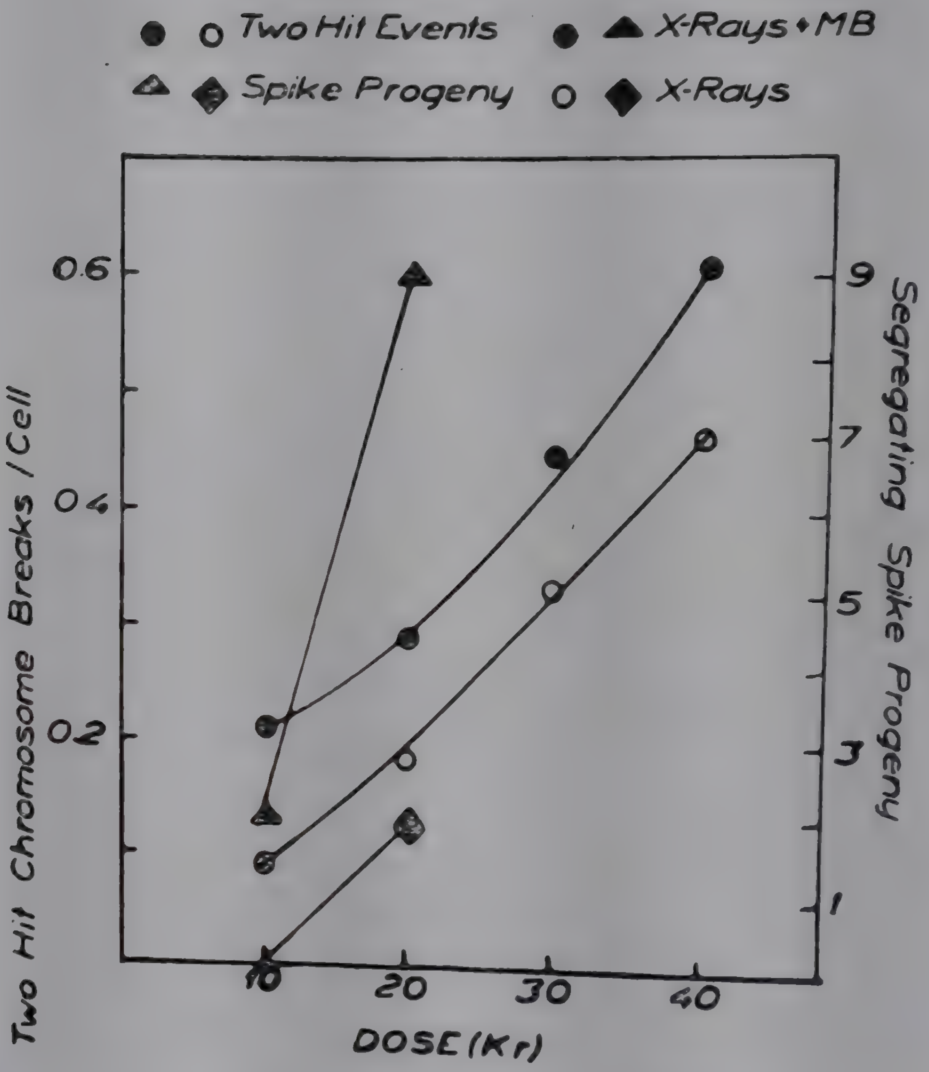

Fig. 10—Modification of response to X-rays of barley seeds, following methylene-blue treatment

of the chromosome composed of DNA and closely associated basic protein (how they are arranged to form the chromosome, is still a puzzle), the long generation time, the cell selection which operates in organized tissues against mutated cells, and the lack of precise scoring techniques, have made this type of study in higher organisms rather difficult[22]. Evidence does exist, however, for such differential mutagenic response in Drosophila[23]. In higher plants, differences in gross proportions of the different mutations induced in barley, have led the Swedish School of mutation workers to postulate some sort of specificity to chemicals in contrast to randomness of action associated with radiations. Favret[24] has presented some evidence of mutagenic specificity, with regard to different loci, pertaining to mildew resistance in barley. The chlorophyll mutations induced by mono-functional alkylating agents like EMS are of special interest. EMS produces a high frequency of chlorophyll mutations; by appropriate treatment the mutation rate can be increased to as high as 100% on M_1 plant basis, about 60% on earhead progeny basis and about 17% on M_2 population basis. This would be about 6 to 8-fold increase over the highest rates obtained with radiations.

There are evidences to suggest that this higher increase in the mutation rate is due to some specificity of this agent for loci responsible for chlorophyll production. When dry seeds are treated with EMS solution, in the first generation itself the leaves show chlorophyll deficient streaks. Since dominant homozygote and heterozygote represent the normal chlorophyll condition, these streaks can arise only if concerned loci are mutated to recessive nature. High incidence of sectors obtained in our experiments could be explained only if we assume that there is a specificity of this mutagen to the chlorophyll controlling loci. Such sectors are produced with great rarity in experiments with radiations, since it is a chance that both the homologous loci will be hit during irradiation of seeds. Swaminathan[14] suggested that the localized breakage at and near the centromere in chromosomes of barley and the incidence of high frequency of chlorophyll mutations in barley following EMS treatments indicate that these loci might be situated near the centromere. Recent data on the linkage map of *Hordeum* (Robertson[25]) support this view, as it is shown that many of these loci are situated near the centromere.

Further support of this mutagen (EMS) having some specificity comes from the data of chlorophyll mutations in hexaploid *Triticum aestivum* which comprises 3 related genomes. Nullisomics (i.e. deficient for a pair of chromosomes) do not show any chlorophyll deficiency[26] indicating that each constituting genome has a set of genes for production of chlorophyll. Thus, to produce a chlorophyll deficient mutant, one has to hit three sets of genes in all the 3 genomes. With radiation, it is very difficult to induce chlorophyll mutations in wheat in M_2 generation. EMS, on the other hand, produces relatively large number of chlorophyll-deficient mutations in wheat[14], indicating that the loci governing chlorophyll production are affected in all the three genomes. Since these genomes are closely related, their genetic structure with regard to chlorophyll development can be assumed to have evolved in a similar way. Thus, the chlorophyll mutations produced in hexaploid wheat suggest that all the three genomes are affected simultaneously probably because of the specificity of the mutagen to these loci. However, many of the characters studied can be altered by several loci, which makes the analysis based on chlorophyll mutation data rather difficult. A critical evaluation of single locus mutations will throw some light on this question. A study with six dominant markers in barley, to evaluate the relative efficiency of various agents for their mutagenicity to the particular locus, is in progress in our laboratory. In hexaploid wheat, majority of the earhead mutations produced by radiations are speltoids; with ethylmethane sulfonate this mutation is very rare, or not induced[14]. With a more critical study and more refined systems, it may be possible to control the mutation process, at least at a few well-refined loci in higher plants.

SUMMARY

The cytological and genetic changes induced by several chemical mutagens, which are known to induce molecular mutations in microorganisms have been studied in higher plants. Some localization points of chromosome breakage have been established which indicate relative richness of adenine-thymine or guanine-cytosine bases at these regions. From the localization of breaks, centromeric regions in most of the cases have been found to be rich in GC pairs and secondary constriction or nucleolar organizing regions in A-T pairs. Several of the monofunctional alkylating agents, base analogues, and photodynamic dyes have been tested for their relative mutagenic ability in barley; the results are discussed in brief, with special reference to directed mutagenesis.

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Studies on the Formation of Liponucleoprotein Complex by Orotic Acid in Albino Rats

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Recent years have witnessed the elucidation of the chemical entities and the sequence of reactions that ends ultimately in the translation of genetic information into polypeptides[1,2]. In microorganisms factors involved in the regulation of enzyme synthesis and activity have been recognized. The popular concept is that the interaction of 'small molecular weight metabolites' with repressors, the primary products of regulatory genes, results either in the acceleration or inhibition of enzyme synthesis. It is presumed that the effects of specific metabolites in inhibiting and stimulating the synthesis of messenger RNA and protein are mediated by allosteric transition of the repressor[4].

It is not clearly known whether these concepts evolved as a result of microbial research are as such transferable to multicellular organisms. Nevertheless, they do suggest that a variety of human diseases may be a consequence of the formation of altered enzyme, altered feedback and altered membrane transport. This investigation is part of our programme to study the basic mechanisms at molecular level underlying the onset of diseases. The results presented in this communication are indicative of the possibility that the expansion of pyrimidine nucleotides pool in the absence of 'feedback' between orotidylic acid and uridylic acid initiates the synthesis of a 'nucleoprotein' in association with lipid in the cytoplasm of the liver. The changes observed appear to induce fatty liver—a condition that precedes liver cirrhosis and necrosis.

MATERIALS AND METHODS

Animals and diets

Young male albino rats weighing 40-50 g. were used in this investigation. The animals were divided into two main groups and kept in individual cages.

One group of rats was given the basal diet of composition described earlier[5] and the second group was given the basal diet containing 1% orotic acid (obtained from Sigma Chemicals, U.S.A.). Unless otherwise stated the rats were given food and water *ad libitum* for three weeks. At the end of the experimental period, the animals were sacrificed. The livers were perfused with $0{\cdot}25M$ cold sucrose solution before analysis. The various radioactive precursors of nucleic acids and protein were injected intraperitoneally 8 hours prior to sacrifice, at concentrations indicated in respective tables. Orotic acid-6-^{14}C, adenine-8-^{14}C, ^{32}P-labelled inorganic phosphate and valine-1-^{14}C were purchased from Atomic Energy Establishment, Trombay; phenylalanine-U-^{14}C, lysine-U-^{14}C, and formate-1-^{14}C were obtained from Radiochemical Centre, Amersham, U.K. Actinomycin D was a gift from Dr K. C. Mezey of Merck-Sharp & Dohme, New York.

Isolation of subcellular fractions

The liver was homogenized in $0{\cdot}25M$ sucrose containing $0{\cdot}0033M$ calcium chloride using a Potter-Elvehjem homogenizer with Teflon pestle. The unbroken cells and cell debris were removed by filtration through cheese cloth. The intracellular particles were separated by differential centrifugation technique[6] using Servall Rc-2 and Spinco Model L preparative centrifuges.

Isolation of protein-nucleoprotein complex

The protein-nucleoprotein complex from the various cellular fractions was precipitated by the addition of trichloroacetic acid (TCA) to give a final concentration of 10% at 2°C. The precipitate was washed thrice with cold 5% TCA to remove the acid-soluble nucleotides. The lipid was removed by washing twice with hot Bloor's mixture once with acetone and once with ether; the residual material was dried at 60°C.

Measurement of radioactivity of protein

A known weight of the finely powdered protein was suspended in 25% alcohol and spread evenly on a stainless steel planchet, dried and the radioactivity determined in a Geiger counter connected to a Panex scaler. The values were corrected for self-absorption and background radioactivity. The data are expressed as c.p.m./g. of fat-free dry powder. The percentage increase or decrease as compared to the control was calculated and is recorded in the tables. The experiments were repeated four times and the same pattern of results was obtained each time. The results of a typical experiment are given in the tables.

Measurement of radioactivity of nucleic acids

A known weight of protein-nucleoprotein complex was hydrolysed with $0{\cdot}3\ N$ potassium hydroxide at 37°C. for 18 hr. The protein and deoxyribonucleic acid (DNA) were precipitated with perchloric acid. The precipitate was removed by centrifugation and the clear supernatant containing ribonucleotides was plated and dried *in vacuo*; the radioactivity was determined as described above.

The amino acids were separated by two-dimensional descending paper chromatography (Whatman No. 1) using the solvent system butanol-acetic acid-water (4:1:5) (upper layer) in one direction and metacresol: phenol : borate buffer (*p*H 8·6) in the second direction. Amino acid spots were located by spraying the chromatogram with ninhydrin (0·2% solution in acetone). The nucleotides were separated by Dowex-1-formate (200-400 mesh × 8). Adenylic acid and cytidylic acid were eluted together using 0·5 *M* formic acid and guanylic acid and uridylic acid with 1·0 *M* formic acid containing 0·5 *M* sodium formate. Using the differential equation given by Zscheile and Munay[7], the concentrations of the four nucleotides were calculated in μmoles taking adenylic acid as 10. The enzymic hydrolysis of RNA by bovine-pancreatic ribonuclease was carried out according to the procedure described by Anfinsen *et al.*[8]

Results and Discussion

Orotic acid administration has been shown to induce a decrease in the level of adenine-containing coenzymes[5,9] and adenine nucleotides and an increase in the pyrimidine nucleotides[10,11]. The results presented in Table 1 reveal that these changes are manifested mainly in the nuclear and supernatant fraction of the liver. The rate of incorporation of adenine-8-^{14}C is not significantly decreased, thereby suggesting that the observed decline of the level of adenine nucleotides was not due to a decrease in the synthetic capacity of the cell. The decrease may be due to a drain towards the formation of the "lipo-nucleo-protein" (LNP) described in the latter section of this communication.

In Tables 2 and 3 are given the results obtained on changes in the level

Table 1—Effect of Orotic Acid Administration on the Level of Acid-soluble Nucleotides in Rat Liver

Fractions	% Increase (+) or decrease (−) as compared to that of control		% Incorporation of adenine-8-^{14}C
	Purine nucleotides	Pyrimidine nucleotides	
Nuclear	−62	+180	+13
Supernatant	−51	+100	− 5

Adenine-8-^{14}C (2μC) was injected intraperitoneally. Animals were killed 8 hours after administration of the labelled precursor.

of RNA and the incorporation of labelled precursors into RNA of the various cellular fractions, respectively. It is evident that the mitochondrial fraction shows a significant fall in the level of RNA. Also, the incorporation of all the precursors tried is reduced appreciably. It may be concluded, therefore, that the decrease of RNA level reported earlier[5] pertains to the mitochondrial fraction of the cell and is due to the inhibition of synthesis. Lowe and Lehninger[12] reported that mitochondria treated with cortisone contained no RNA. Leakage of mitochondrial constituents is known to occur in carbon tetrachloride poisoning[13]. Results of the present investigation do not favour such a possibility. It is, however, very difficult to explain at present the specific decrease of mitochondrial RNA. The supernatant fraction of liver exhibits a slight increase in both the level and the synthesis of RNA as judged by the rate of incorporation of adenine-8-^{14}C which may represent a high turnover of terminal end of S-RNA. This is understandable considering the increased incorporation into, and synthesis of "LNP", to be described later.

TABLE 2—Changes in the RNA Levels of the Various Subcellular Fractions of Rat Liver on Orotic Acid Administration

Fractions	% Increase (+) or decrease (−) as compared to that of control
Nuclear	No change
Mitochondria	−42
Microsomes	− 6
Supernatant	+10

TABLE 3—Effect of Orotic Acid on the Incorporation of Radioactive Precursors into the RNA of the Various Sub-cellular Fractions from Liver

Precursor injected	% Increase (+) or decrease (−) as compared to that of control			
	Nuclei	Mitochondria	Microsomes	Supernatant
Orotic acid-6-^{14}C	−63	−79	−85	−63
Adenine-8-^{14}C	+40	−27	+43	+110
Formate-1-^{14}C	No change	−33	No change	+ 2
Phosphate-^{32}P	−33	−24	−37	+22

The precursor (2μC) was injected intraperitoneally and the rat was killed after 8 hours.

Nuclear and microsomal fractions exhibit no change in the levels of RNA. The decrease observed in the incorporation of orotic acid-6-^{14}C can be attributed to an enormous dilution caused by an expansion of pyrimidine nucleotides pool. Even at the base level, orotic acid-6-^{14}C administered will get diluted. The increased incorporation of adenine-8-^{14}C shows that the animal is kept under an acute state of adenine deprivation. Hence, whatever little quantities of adenine get into the system are rapidly utilized to meet the metabolic need. It is pertinent to mention that the reversal of the effects of orotic acid begins to occur a few hours after the administration of adenine. The diminished incorporation of ^{32}P phosphate is confined to adenylic acid and guanylic acid moieties of RNA molecule. These results provide convincing evidence for the synthesis of nucleic acid with altered base composition in the orotic acid-treated rat.

In Table 4 are given the data on the incorporation of amino acids into protein. It is interesting to note that no alteration occurred in the level of protein in the sub-cellular fractions, excepting the appearance of the LNP in the cytoplasm. Among the amino acids studied, an increase in the incorporation of only phenylalanine and tryptophane was noticed. The increase of phenylalanine in the nuclear and supernatant fractions was quite striking. Lysine incorporation showed no change except for a decrease in mitochondria and an increase in supernatant. The incorporation of valine decreased significantly in all the fractions. These differences in the rate of incorporation of different amino acids indicate an alteration in the nature of protein synthesized as a consequence of the formation of altered nucleic acid in the animal. Increased incorporation of phenylalanine may be due to the synthesis of nucleic acid with predominant uridylic acid content. *In vitro* experiments carried out in bacteria have revealed the stimulation of synthesis of polyphenylalanine by polyuridylic acid[14,15]. The purine analogue 8-azaguanine has been shown to stimulate the uptake of serine and alanine[16] and inhibit that of methionine and cysteine[17]. The addition of 5-fluorouracil

TABLE 4—EFFECT OF OROTIC ACID ON THE INCORPORATION OF LABELLED AMINO ACIDS INTO THE PROTEIN OF VARIOUS SUB-CELLULAR FRACTIONS FROM LIVERS

Amino acid injected	% Increase (+) or decrease (−) as compared to that of control			
	Nuclear	Mitochondria	Microsomes	Supernatant
Phenylalanine	+75	+ 5	+30	+47
Valine	−40	−38	−42	−63
Lysine	No change	−34	−12	+29
Tryptophane	+46	+17	+47	+51

The amount of radioactivity injected (intraperitoneally) was 2μC, 5μC, 2μC and 5μC in the case of the four amino acids, respectively.

to the exponentially growing *Esch. coli* cells has been found to inhibit the incorporation of proline and tyrosine in *Esch. coli* and *B. megaterium*[18]. This effect was related to a diminished binding of labelled amino acids to S-RNA and an alteration in the composition of this RNA[18]. No such information is available in mammalian systems.

Detection and partial characterization of "lipo-nucleo-protein" (LNP) in rat liver cytoplasm

During the isolation of the various subcellular fractions by differential centrifugation "a fluffy white material" was always observed in the topmost layer of the homogenized material. This was carefully skimmed and resuspended in 0·25 M sucrose and centrifuged to free it from the adhering cellular particles; the process was repeated with 5% TCA three to four times. Examination under polarizing microscope revealed no nuclear contamination. The material was then washed thrice with hot Bloor's mixture and once with acetone and ether respectively and dried in air. About 75-125 mg. of solid material in a fine powder form was obtained from 5-8 g. of liver. Yield of this material depended on the amount of orotic acid administered. Livers of normal rats had no such material. The concentration of alcohol-ether-acetone washings yielded appreciable quantities of lipid. The solid material gave positive test for ribonucleic acid and protein.

In Table 5 are given data on the incorporation of amino acids into the protein of this material. It is observed that among the four amino acids studied the incorporation of phenylalanine and tryptophane into this particular compound is far greater than that observed in other cellular fractions. Two-dimensional paper chromatography of the acid-hydrolysed protein revealed the presence of glutamic acid, aspartic acid, serine, glycine, threonine, alanine, lysine, valine, tryptophane, histidine, arginine, phenylalanine, proline, tyrosine and methionine. The electrophoretic mobility of the protein as studied by paper electrophoresis (carried out at 8 V./cm. for 6 hr using 0·1 M phosphate buffer of pH 7·4) was -2 cm. On agar gel electrophoresis (in veronal buffer, pH 8·6, for 6 hr at 13 V./cm.) the protein moved a distance of $+3·5$ cm. Further, this protein moved as a single band both in paper electrophoresis and in agar gel electrophoresis.

In Table 6 are presented data on the incorporation on nucleic acid precursor into this material. Both purines and pyrimidine precursors were incorporated and the rate of incorporation, particularly of orotic acid-6-^{14}C into LNP was significantly more than in any other cellular fraction. Alkali hydrolysis and separation of the liberated 2′, 3′-nucleotides revealed adenylic, guanylic, cytidylic and uridylic acids. The radioactivity, when orotic acid-6-^{14}C was used as the marker, was completely released by the action of RNase. Chromatography of the reaction mixture after enzyme digestion showed the release of uridylic acid and cytidylic acid.

In Table 7 are given the results of incorporation of orotic acid-6-^{14}C

TABLE 5—Incorporation of Amino Acids into Lipo-nucleo-protein (LNP) of Orotic Acid-treated Rats

Amino acid injected	c.p.m./g. of LNP
Phenylalanine	20,000
Lysine	6,400
Valine	8,000
Tryptophane	25,000

The amino acid (2μC) was injected intraperitoneally and the rat was sacrificed 8 hours after administration of the amino acid.

TABLE 6—Incorporation of Nucleic Acid Precursors into Lipo-nucleo-protein (LNP) of Orotic Acid-treated Rats

Precursor injected	c.p.m./g. of LNP
Orotic acid-6-^{14}C	10,000
Adenine-8-^{14}C	24,000
Formate-1-^{14}C	11,550

The precursor (4μC) was injected intraperitoneally and the rat was sacrificed 8 hours after administration.

TABLE 7—Incorporation at Different Time Intervals of Orotic Acid-6-^{14}C into Lipo-nucleo-protein (LNP) and other Sub-cellular RNAs from Livers of Orotic Acid-fed Rats

c.p.m. recovered in	1 hr	2 hr
Nuclei	50	98
Mitochondria	Negligible	92
Microsomes	Negligible	108
Supernatant	24	110
LNP	44	130

into the material at different time intervals. The compound is labelled even at 1 hr after administration of the precursor, the time at which mitochondria and microsomes have very little radioactivity; the extent of labelling was more than in the supernatant fraction. Two hours after administration of orotic acid-6-^{14}C all the cellular particles were labelled; however, the labelling of the compound was significantly more than that of other cellular fractions. These results suggest that the nucleic acid of LNP is rapidly synthesized, indicating a high turnover rate.

The effect of actinomycin D on the incorporation of orotic acid-6-^{14}C into LNP and nuclear RNA is recorded in Table 8. Actinomycin D inhibited the synthesis of nuclear RNA as well as the nucleic acid of LNP as judged by the incorporation of orotic acid-6-^{14}C. It has been suggested that the entire complement of RNA (nuclear, ribosomal and transfer) of bacterial and mammalian cells is produced by an actinomycin D-sensitive and DNA-dependent reaction[19,20]. The synthesis of the nucleic acid part of the LNP also appears to require continuous nuclear function.

In Table 9 is given the nucleotide composition of nucleic acid isolated from LNP by the method of Kay and Dounce[21]. The results presented in the table are from two different preparations. Although there is a slight variation in the absolute figures of the nucleotides, the pattern of distribution of the nucleotides in the RNA molecule remains the same. The concentration of uridylic acid and cytidylic acid predominates over that of adenylic and guanylic acids.

TABLE 8—EFFECT OF ACTINOMYCIN D ON THE INCORPORATION OF OROTIC ACID-6-^{14}C INTO THE RNA OF SUB-CELLULAR FRACTIONS AND LIPO-NUCLEO-PROTEIN (LNP) FROM LIVERS OF OROTIC ACID-FED RATS

Fraction	c.p.m./g. nucleoprotein	
	Control	Actinomycin D-treated
Whole homogenate	6,150	1,000
Nuclear	5,650	1,000
LNP	10,000	2.000

Actinomycin D was injected at a concentration of 2 mg./kg. body weight intraperitoneally.

TABLE 9—NUCLEOTIDE COMPOSITION OF LIPO-NUCLEO-PROTEIN FORMED IN OROTIC ACID-FED RATS

Fraction	AMP	GMP	UMP	CMP	Pu/Py	GU/AC	GC/AU
Nuclei	10	12·7	12·2	14·3	0·84	1·01	1·23*
Mitochondria	10	16·9	11·0	14·6	1·03	1·11	1·52*
Microsomes	10	17·9	9·9	15·1	1·03	1·03	1·76*
Supernatant	10	17·3	9·8	17·1	1·04	1·02	1·71*
LNP I	10	14·08	28·45	28·31	0·424	1·103	1·11
LNP II	10	14·77	18·69	19·54	0·647	1·196	1·132

*Literature values[25].

It has been suggested by Scholtissek[22] that RNA of high turnover rate may be rich in uridine nucleotides. The experiments of Allfrey and Mirsky[23] also indicate that the uridine containing nucleotides of nuclear RNA are labile and participate in a rapid turnover reaction. It has been reported that the specific activity of RNA is always higher with orotic acid as the marker than with 4-amino-5-imidazole carboxamide[24]. The cellular RNA of rat liver is of the GC type; messenger RNA is shown to have the base composition similar to DNA. No RNA has so far been known to be UC type. The appearance of such a nucleic acid in the cytoplasm of the liver of orotic acid-treated rats is of interest since such an observation has not so far been recorded in the literature.

Experiments demonstrating either the accumulation of "a typical RNA" or alteration in the composition of the nucleic acid have been done so far only in unicellular organisms. Further, such effects in these organisms are caused either by chloramphenicol or base analogues like 5-fluorouracil and 8-azaguanine. To-date no literature is available wherein a normal metabolite such as orotic acid has been shown to cause the formation of an altered RNA in combination with protein and lipid in the cytoplasm of rat liver.

The sequence of reactions that can be formulated on the basis of the above discussion are (i) an expansion of the pyrimidine nucleotides; (ii) synthesis of nucleic acid with a high content of pyrimidine nucleotides; (iii) release of this nucleic acid into the cytoplasm; and (iv) its accumulation in the cytoplasm in combination with protein and lipid. This scheme is only of a tentative nature requiring extensive experimental study. The results so far obtained indicate the reversal of the "orotic acid fatty liver" by actinomycin D. In conclusion it may be stated that fatty liver produced by feeding orotic acid appears to be the end result manifested by an initial alteration in the composition of nucleic acid.

SUMMARY

Evidence has been presented as to the presence of a lipo-nucleo-protein (LNP) in the cytoplasm of the liver of rats treated with orotic acid. The nucleic acid moiety of this LNP contained more uridylic and cytidylic acids than adenylic and guanylic acids. The base composition does not resemble that of RNA of any of the cellular fractions. Actinomycin D is found to inhibit the incorporation of orotic acid-6-^{14}C into the nucleic acid of LNP. Certain changes such as an expansion of pyrimidine nucleotides pool, alteration in the base composition of cellular nucleic acids (particularly the nuclear and supernatant fractions), and a differential rate of utilization of amino acids for protein synthesis have been observed following administration of orotic acid to rats.

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Mechanism of DNA Synthesis

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Although great progress has been made in the field of DNA synthesis, and Kornberg's polymerase has had striking success in copying a large number of DNA primers, the exact process of DNA replication in a living cell is not well understood today. Since the assignment of a double helical structure to DNA by Watson and Crick[1], many attempts have been made to study the mechanism of DNA replication. The classical experiment of Meselson and Stahl[2] indicates that parental DNA is equally divided between two subunits, which remain intact through many generations during DNA replication. These two subunits have been identified as the two strands of DNA double helices[3]. The recent calculations of the outer limits of the time required for unwinding a 10 million molecular weight DNA are 1·54 sec. and 150 days respectively[4]; for a 120 million molecular weight DNA, the lower time limit is 12·4 min.[5] which is longer than the time observed for a single replication of T_2 DNA. The feasibility of unwinding such a long DNA molecule appears to be extremely difficult to conceive. Realizing this difficulty, Dounce[6] suggested a long time ago the possibility of synthesizing a single deoxyribopolynucleotide chain on DNA double helix, and the possibility of the newly synthesized strand to act as a template for the synthesis of complementary chain.

Recently, Bollum[7], using purified calf thymus polymerase, demonstrated that only single-stranded DNA can act as a primer for DNA synthesis. T_2 phage induced polymerase also requires single-stranded DNA[8]. The recent work of Kornberg and his colleagues[8a] who used a very highly purified (20,000 times) polymerase, obtained from *Escherichia coli* and *Bacillus subtilis*, also indicates the ability of two-stranded native calf thymus DNA and heat denatured DNA to act as a primer in DNA synthesis. Many investigators have, on the other hand, observed much greater priming activity with two-stranded DNA than with heat-denatured DNA in the systems involving the use of high speed supernatant fractions prepared from either young rat liver, regenerating rat liver or mouse leukemic cells[9–12]. Whether two-stranded DNA or heat-denatured DNA can act more effectively as a primer seems to depend largely on the polymerase preparations. As the enzyme is progressively purified, the single-stranded DNA becomes a better primer in DNA synthesizing system. If it is the single-stranded DNA that only can act as a primer, it is reasonable to expect that in such crude systems as mentioned above, the two deoxyribopolynucleotide chains of DNA template are first separated, and then each of the two strands acts as a primer. Such a possibility was recently investigated in our laboratory[13]. For this purpose, two-stranded, native calf thymus DNA was incubated with soluble extract of regenerating rat liver in the presence and absence of Mg^{2+} with or without EDTA, at 37°C. for 30 min. The DNA was reisolated, using the conventional method of Kay and Dounce[14]. The inability of this DNA to act as a primer in Bollum's system and its ability to act as a primer on heat-denaturation, as shown in Fig. 1, might be considered to indicate that the separation of the two deoxyribopolynucleotide chains of the primer DNA prior to DNA synthesis, is not a prerequisite condition for DNA replication. The reisolated DNA, however, exhibited considerably higher (200%) priming activity than the native DNA in the system involving the use of high speed supernatant fraction of regenerating rat liver. These results are illustrated in Table 1.

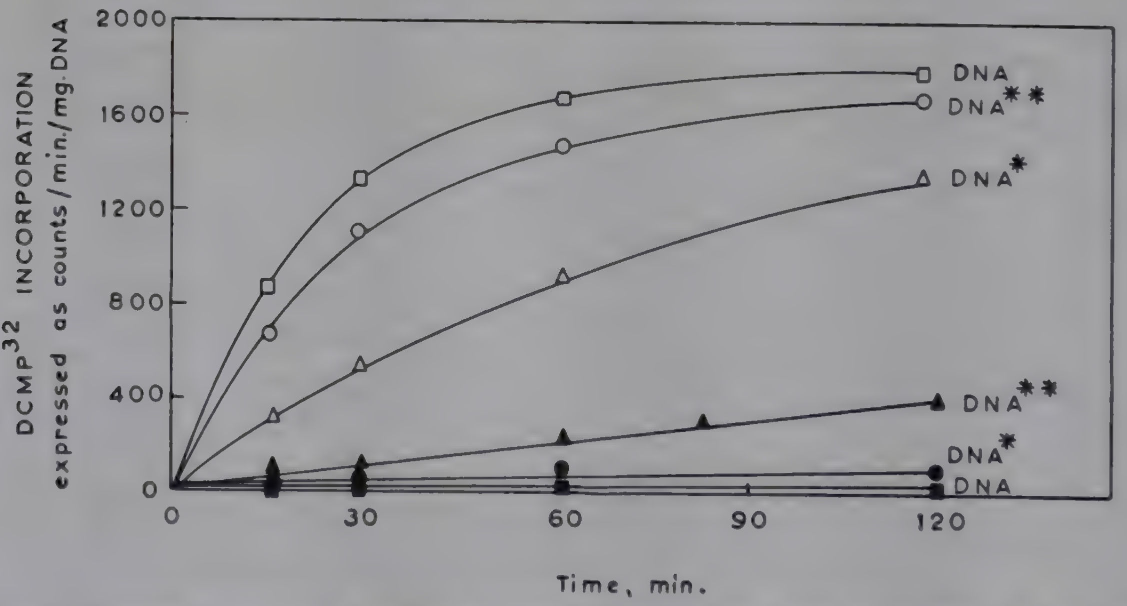

Fig. 1—Priming activities of DNA samples before and after heating: The reaction mixture in a final volume of 0·2 ml. contained the following: 25 mμ moles of dATP, dCTP, dTTP each; 15·0 mμ moles of dCTP labelled with ^{32}P in the ester phosphate (specific activity of dCMP was 2790 c.p.m./mμ mole), 2 mmoles of mercaptoethanol, 125 μg. of enzyme protein and 25 μg. of DNA. Incubation time was 30 min. at 37°C. 25 μl. of the reaction mixture was used for estimation of dCT^{32}P incorporation by the paper disc procedure. DNA* is the reisolated DNA from the incubation mixture containing EDTA. DNA** is the reisolated DNA from the incubation mixture where EDTA was omitted. ○—○, △—△, □—□ Priming activity of DNA samples before heating. ●—●, ▲—▲, ■—■ Priming activity of DNA samples after heating.

TABLE 1—ACTIVITY OF VARIOUS DNA SAMPLES IN REGENERATING RAT LIVER SYSTEM

[The values given are percentages of the value for unheated calf thymus DNA, the activity of which was taken to be 100. The reaction mixture contained in a total volume of 1 ml., the following: 1·0 mg. of DNA, 20 μmoles of ATP, 0·02 μmoles of dCMP, dAMP and dGMP each, 8 μmoles of 3-phosphoglycerate, 2·5 μmoles of Mg^{2+}, 20·0 μmoles of Tris buffer (*p*H 8·0), 0·056 μmoles of ^3H-thymidine (equivalent to $0·88 \times 10^5$c.p.m.) and 0·5 ml. of high speed supernatant fraction. Incubation time was 30 min. at 37°C. DNA was isolated by the method of Schmidt and Thannhauser and the tritium content assayed in a windowless gas flow-counter. The amount of DNA synthesized in 30 min. was measured as the incorporation of ^3H-thymidine/mg. of DNA[13]]

Types of DNA	Before heating	After heating
Calf thymus DNA	100	45-55
DNA*	240-290	38-46
DNA**	160-180	42-47
Calf thymus DNA+high-speed supernatant fraction preincubated for 30 min. at 37°C. before other reagents were added	220-250	

We have also recently noted[15] that two-stranded DNA acts more efficiently as a primer than the heat-denatured DNA, when soluble extract of young rat liver serves as the source of DNA polymerase; the ability of two-stranded DNA to act as a primer decreases from birth through maturity, while the ability of the heat-denatured DNA to act as a primer increases steadily with age, although its activity is very low in young rats to begin with. It should also be noted that when the livers of 4-6 months old rats are used, the incorporation of thymidine into primer DNA is the same whether the primer is two-stranded DNA or heat-denatured DNA; thus two-stranded DNA and heat-denatured DNA can be equally utilized as a primer by rats of this age.

All this information might be considered to suggest that DNA synthesis starts at the end of the double helix where the bases are not hydrogen bonded, as far as we know. These bases are free to form hydrogen bonds with the incoming bases, purine or pyrimidine, satisfying, the A-T, G-C rule. This is followed by further successive breakdown of hydrogen bonds between the pair of bases next in the row and is accompanied by the formation of new hydrogen bonds between the unpaired bases with the incoming bases. This stepwise process continues until two new double-stranded DNA molecules are formed from one two-stranded primer DNA. It is assumed that stepwise breakdown of hydrogen bonds between the bases, and the synthesis of DNA occur concomitantly.

Mantavinos and Canellakis[9] using a cell-free preparation from mouse leukemic cells found a five-fold increase in the incorporation of ^{32}P-TMP into two-stranded DNA in the presence of DNase I. Similarly, DNase I-treated reisolated DNA has been found to be four times more efficient as a primer for DNA synthesis in the system involving the use of high speed supernatant fraction prepared from regenerating rat liver[10]. The rate of cell division of a variety of cells has been shown to be greatly enhanced by the addition of a small amount of DNase I[16]. The ability of DNase I to increase the rate of mitosis in sea-urchin eggs has also been recently reported by Zahn[17]. When all these observations are considered together with the recent findings of Devi *et al.*[18,19] on the variation of DNA concentration and DNase activity in the liver of rats with age, beginning from infancy to senility, one may begin to think of the possibility of DNAse's participation in DNA synthesis. It should be recalled that Allfrey and Mirsky[20] also suggested such a possible role of DNase II. We do not know whether DNA molecule as a whole, a part of it or smaller fragments of it, act as a template for DNA and RNA synthesis. If in a living cell, fragmented parts of DNA with appropriate chain length, act as primers for DNA synthesis, it is quite possible that DNase I might have some role in DNA synthesis. It was, therefore, felt desirable to investigate the ability of degraded DNA to act as primer in DNA synthesis, and to know how the genetic recombination takes place between the subunits after their replication. The effects of DNase I and sonication on the priming efficiency of two-stranded and heat-denatured DNA in DNA synthesis were investigated in a system involving the use of soluble extract of young, old or hepatectomized rat liver, and the results are presented here.

It is known that if DNA is exposed to the action of different amounts of DNase for the same length of time, it undergoes degradation without denaturation to smaller fragments of various chain lengths, having different molecular weights[21,22]. Similarly when DNA is exposed to sonic vibration for different lengths of time, it undergoes degradation to various fragments. They all differ in molecular weight[22]. In this way, a series of homologous DNA samples are prepared. They all retain double-helical structure, as far as can be judged from the shape of their melting point curves shown in Fig. 2. They appear to be very much similar to the melting point curve of the parental two-stranded, undegraded calf thymus DNA, and their melting points also appear to be identical.

The action of DNase on DNA synthesis was studied by adding different amounts of DNase to the assay system before the addition of high speed supernatant fraction of rat liver. In the second series of experiments, all the samples of DNA, obtained after exposing the native, two-stranded DNA to sonication for different lengths of time, were separately used as primers in the systems involving the use of high speed supernatant fraction of young or old rat liver. Rats of 3-4 weeks and 14-16 months of age were used for this purpose. In each case, the animal was sacrificed by decapitation, the liver was immediately removed and chilled in ice, and a 20% homogenate was prepared in a Dounce homogenizer at 0°C. in a medium containing 0·25 *M* sucrose, 0·004 *M* $MgCl_2$ and 0·035 *M* $KHCO_3$. The high speed

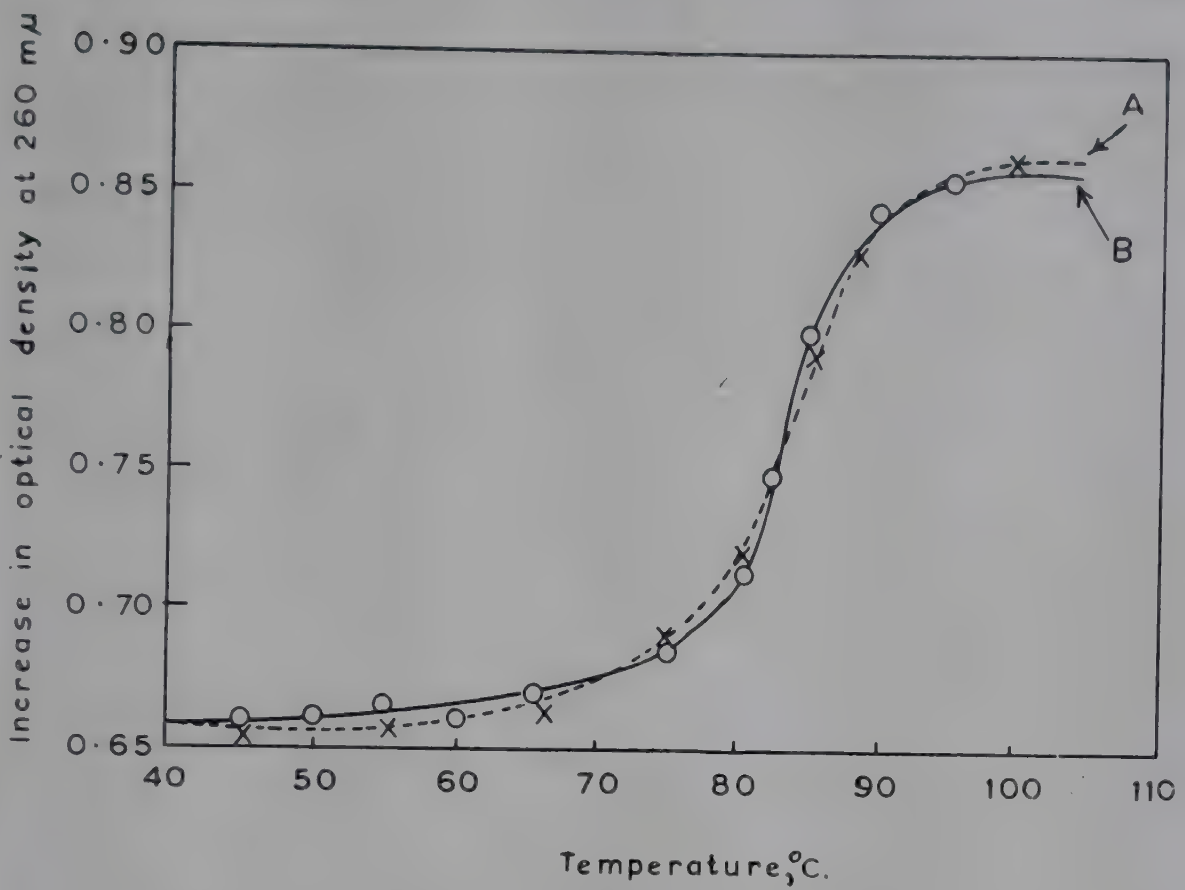

FIG. 2—OPTICAL DENSITY-TEMPERATURE PROFILES OF NATIVE TWO-STRANDED CALF THYMUS DNA (o—o) AND DNA EXPOSED TO SONIC VIBRATION FOR 2 min. (x—x)

supernatant fraction, used as the source of polymerase activity, was prepared by centrifuging the homogenate at 105,000 *g* (Spinco, Model L) for 2 hr.

The incorporation of 2-^{14}C-thymidine (2- ^{14}C-TMP was also used in some experiments) into primer DNA was studied in a system containing the high speed supernatant fractions prepared from the livers of young, old and hepatectomized rats, Mg ions, triphosphates of the four deoxyribonucleosides (dGTP, dCTP, dTTP, dATP), ^{14}C-thymidine or ^{14}C-TMP, and a primer DNA. The assay method involves the incorporation of ^{14}C-TMP into primer DNA. The results obtained can be summarized as follows[23].

The results of Table 2 show that the addition of DNase to the assay system results in an increase in the incorporation of ^{14}C-TMP into added primer DNA. This effect of DNase is observed when two-stranded DNA or heat-denatured DNA acts as a primer, in the system involving the use of soluble extract, prepared either from young or old rat liver, as the source of polymerase. It is interesting to note that the two-stranded DNA which acts as a poor primer when soluble extract from adult rat is used as polymerase source, becomes a better primer in the same system if its activity is measured in the presence of 20 μg. of DNase. It should, however, be pointed out that although the priming activity of heat-denatured DNA is considerably enhanced when measured in the presence of DNase, the amount required is much less (50%) than is needed for maximum ^{14}C incorporation when two-stranded DNA is used as a primer. It should be noted that if the concentration of DNase is increased above 20 μg. in the case of two-stranded DNA primer and 10 μg. in the case of heat-denatured DNA, their priming efficiency falls sharply.

If the priming efficiencies of the various homologous samples of DNA, obtained by exposing the latter to sonic vibration for different lengths of time are compared, it becomes evident that DNA exposed to sonication for only 2 min. acts most efficiently as a primer; in other words, maximum incorporation of ^{14}C-TMP occurs into fragmented DNAs when they are of only certain specific chain length. The molecular weight of these subunits as determined by light scattering method as described by Cavalieri and Rosenberg[21] is roughly one million. The results obtained are illustrated in Table 3. The results of Table 3 also demonstrate that if DNA is exposed to sonication for more than 2 min. or less, the amount of ^{14}C incorporated into primer DNA declines rapidly, as has been previously observed when DNA is subjected to the action of DNase at any concentration other than 20 μg. However, when DNA is first exposed to the action of sonic vibration for less than 2 min. and then subjected to the action of 20 μg. of DNase, the incorporation of ^{14}C-TMP into the fragmented DNA, is further augmented. The amount of ^{14}C incorporated exceeds the value that can be obtained when DNA is subjected either to the action of 20 μg. DNase or exposed to sonic vibration for only 2 min. If the concentration of DNase or the time of exposure of sonication is increased, the incorporation of C^{14}-thymidine into fragmented DNAs also shows a sharp decline.

TABLE 2—EFFECT OF DNASE ON THE INCORPORATION OF ^{14}C-THYMIDINE INTO TWO-STRANDED AND HEAT-DENATURED DNA, CATALYSED BY SOLUBLE EXTRACTS OF YOUNG AND OLD RAT LIVER

[The incubation mixture contained in a volume of 0·4 ml., 25 μmoles dATP, dCTP and dTTP; 2-^{14}C-thymidine (10,000 c.p.m.); 2m moles of mercaptoethanol; 8·0 μmoles of magnesium acetate; Tris-acetate (75 μmoles; *p*H 8·5); 350 μg. of calf thymus DNA; Ca^{2+} (25 μmoles); and 2·5 mg. of enzyme protein incubation period was 1 hr at 37°C. DNA polymerase assay was carried out by the disk procedure[23]]

Amount of DNase added	Soluble extract of 3-4 weeks old rat liver		Soluble extract of 10-12 months old rat liver	
	Two-stranded DNA	Head-denatured DNA	Two-stranded DNA	Heat-denatured DNA
μg.	c.p.m./ml.		c.p.m./ml.	
0	2272	808	832	1640
10	2196	2288	1688	3448
20	4288	1556	3208	2008
50	448	688	144	280
100	168	364	..	..

TABLE 3—EFFECT OF SONIC VIBRATION ON THE PRIMING EFFICIENCY OF TWO-STRANDED DNA IN THE PRESENCE AND ABSENCE OF DNASE IN DNA-SYNTHESIZING SYSTEMS INVOLVING THE USE OF SOLUBLE EXTRACT OF YOUNG AND OLD RAT LIVER

[Conditions of the experiment were the same as given in Table 2]

Duration of sonication sec.	Soluble extract of young rat liver		Soluble extract of old rat liver	
	Without DNAse	With DNAse (20 μg.)	Without DNase	With DNase (20 μg.)
	c.p.m./ml.		c.p.m./ml.	
0	2346	4163	620	1994
30	2674	3218	790	2554
60	2853	5800	1200	2674
120	3340	2656	1888	2220
180	2116	1973	985	720

All this information seems to indicate that degraded DNA with an approximate molecular weight of one million, and having a double helical structure, can act most efficiently as a primer in DNA synthesis. It is conceivable that in a living cell, DNA possessing a molecular weight of six million or more, first undergoes a limited amount of degradation; the subunits thus formed act as primers. If the cleavage of phosphate diester bonds in DNA molecule is non-random, it is possible to conceive that the newly synthesized, incomplete DNAs (subunits) of similar chain lengths, can also recombine through some discrete sites in the DNA molecule. Bernardi *et al.*[24] also postulate the presence of such discrete sites in DNA molecule. These sites are susceptible to the degradative action of DNase. DNase thus appears to have two functions. It first splits the DNA at some specific sites, facilitating thereby the production of subunits with lower molecular weight; secondly DNase I action on DNA may be presumed to yield a certain amount of single-stranded segments and possibly "frayed ends" of the transected DNA molecules that could act as templates in the synthesis of incomplete DNA molecule. It should be mentioned that the recent work of Kozinski *et al.*[25] on the estimation of newly synthesized DNA indicates that if bacteria (*Esch. coli* B) receive chloramphenicol 7-9 min. after T_4 infection, the amount of DNA synthesized is 4 to 5 times more than that synthesized if chloramphenicol is added within 5 min. after infection. The author suggested that the increased synthesis of DNA is due to an increased production of DNase which degrades DNA into smaller subunits which act as primers. Once the synthesis of DNase is blocked, fragmentation of DNA is prevented, and consequently less DNA is synthesized. It thus appears that there are numerous references in the literature to the involvement of fragmentation of DNA in DNA synthesis.

One of the possible questions that can be raised against the assignment of such a role of DNase in DNA synthesis in a living cell is: how the newly synthesized DNA regains the genetic code. One possibility is as suggested by Veldhinsew *et al.*[26] in the case of T_4 DNA. Their work indicates that the subunits of molecular weight of 5×10^5 or 1×10^6 that are formed either by the limited action of DNase on T_4 DNA or by exposing it to the action of sonic vibration, can still retain the biological activity of the parental DNA. It may also be possible as mentioned above, that the newly synthesized incomplete DNA molecules (subunits) get connected with each other, through the same discrete sites of the DNA molecule which were previously hydrolysed by DNase. Working with $\phi \times 174$ and T_4 bacteriophage-infected *Esch. coli* cells, Kozinski[27], on the basis of his findings, suggested that DNA replication in these cases should also include, in addition to the semi-conservative mechanism of DNA replication, another mechanism which allows for fragmentation of the parental phage DNA and its redistribution to the progeny phage. The results of Meselson and Weigle[28] and those of Kellenberger *et al.*[29] also indicate the involvement of a process of fragmentation and physical exchange of transected sections of DNA, during genetic recombination of phage.

The experimental results obtained so far in this laboratory together with the observations made in other laboratories as cited above, allow us to conclude that the best possible mechanism of DNA replication should include a process of fragmentation of DNA to specified chain length (molecular weight), the semi-conservative mechanism of replication of these subunits and the recombination of the new synthesized fragmented DNAs (subunits) through some discrete sites. It is admitted that all the evidences so far accumulated, do not warrant a conclusion, but it is hoped that future experiments to be undertaken, may substantiate the idea incorporated in the theory. One thing should be borne in mind that DNase I is widely distributed in nature; it is present in all organs; its activity also undergoes variation with age just as the DNA concentration in rat liver changes with age. The ability of rat liver to use two-stranded DNA as a primer also varies with age. The occurrence of DNase and the presence of DNA polymerase in the nucleus of a cell where DNA is found, have also been recently reported by Keir *et al.*[12] Considering all the facts together, one can at least formulate a working hypothesis of the possible role of DNase I in DNA synthesis. Further experiments are now under way towards establishing the validity of such a concept.

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Formation of Ribosomes in *Escherichia coli*

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In spite of the crucial importance of ribosomes in protein synthesis, their structure and formation are poorly elucidated. Ribosomal RNA (rRNA) of *Escherichia coli* consists of two distinct components with sedimentation coefficients of 16S and 23S. There is some evidence suggesting that they are synthesized on separate cistrons of the bacterial chromosomes[1–3]. However, characterization of ribosomal proteins has lagged much behind that of rRNA, and we are entirely ignorant as to how their formation is regulated in the cell.

We have recently launched studies on the formation of ribosomes with the belief that structural organization of ribosomes in relation to its functional differentiation into their two subunits will not be understood without pertinent knowledge concerning the formation of their components and the process of their integration into mature particles. The results obtained during the initial phase of our investigation have just been published[4,5].

MODIFIED SHIFT-UP CULTURE FOR PREFERENTIAL SYNTHESIS OF rRNA

Experiments for ribosome formation are complicated by the presence in the cell of messenger RNA (mRNA) which is of fairly high molecular weight, loaded on the ribosomes, and labelled very rapidly upon exposure to isotopic precursors. It was felt essential first to establish culture conditions under which formation of ribosomes is allowed to proceed without concomitant synthesis of mRNA. It was soon found that transfer of starved bacteria to enriched medium beautifully met this requirement[4].

Bacteria grown in a synthetic Tris-glucose-salt medium were held as a heavy suspension (8×10^{10} cells/ml.) in a Tris-salt medium without glucose for 50 min. at 37°C. and then diluted 200-fold with peptone broth. Upon incubation of such a bacterial suspension under vigorous aeration, synthesis of RNA was started immediately but synthesis of protein was

TABLE 1—NUCLEOTIDE COMPOSITION OF RNA SYNTHESIZED IN MODIFIED SHIFT-UP CULTURE*

Type of labelling		Type of RNA	Mole %				Purine	A+U
Start† Min.	Duration sec.		Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	Pyrimidine	G+C
6	15	Total	22·1	33·1	21·6	23·2	0·81	0·85
6	60	Total	23·3	31·7	22·1	22·9	0·82	0·83
6	60	16-23S	23·2	32·3	22·3	22·4	0·80	0·84
		4-14S	23·2	30·2	22·0	24·6	0·87	0·91
6	600	Total	24·7	31·4	22·0	21·9	0·78	0·87
45	60	Total	24·0	26·0	24·8	25·2	1·00	0·97
..	..	rRNA	24·1	31·9	21·5	22·8	0·79	0·88
..	..	mRNA-IV	25·0	25·1	24·4	25·6	1·00	1·02

*Data taken from Mitsui *et al.*[4]
†Interval (in min.) after transfer to shift-up medium.

strongly depressed during the first 15 min. and then slowly resumed with increasing rates. Such experimental conditions may be called here as the modified shift-up culture or simply the shift-up culture.**

Under conditions for steady growth, nucleotide composition of short-pulse-labelled RNA as determined by distribution of ^{32}P resembled that of DNA[6]. By contrast, the composition of short-pulse-labelled RNA during the initial period (0-16 min.) of modified shift-up culture was similar to that of rRNA; at a later stage (45 min.) it approached that of DNA (Table 1). RNA of the early "shift-up" cells was labelled for 15 sec. or 1 min. with ^{32}P, (^{3}H) uridine or (^{14}C) uracil and extracted with Duponol-phenol method[6]. Analyses of such RNA preparations by sucrose density gradient centrifugation revealed that the newly formed RNA mainly consisted of two peaks which roughly coincided with peaks of two rRNA components (16S and 23S)

**The following procedure was followed in all the experiments: The content of reaction mixture was essentially according to Nierenberg and Matthaei[20]. Total volume was 0·25 ml. Samples were incubated at 37°C. for 30 min., and deproteinized with 10% hot trichloroacetic acid (TCA). The precipitates were collected and washed on millipore filters and counted in a gas-flow counter. rRNA was prepared as follows: *Esch. coli* cells from exponentially growing culture were ground with quartz sand and extracted with 3 volumes of 10 m moles Tris-buffer (*p*H 7·8)-0·1 m moles Mg^{2+} containing 2 μg./ml. of DNase and 1 mg./ml. of bentonite. Extract was centrifuged at 10,000 r.p.m. for 20 min. The supernatant was centrifuged at 105,000 *g* for 2 hr in a 3 ml. adaptor tube of Spinco L ultracentrifuge. The pellet was again washed with 10 m moles Tris-0·1 m moles Mg^{2+} containing 1 mg./ml. bentonite, at 105,000 *g* for 2 hr rRNA was prepared from the washed ribosomes by Duponol-phenol method[6].

with a slight shift of the peak position (Fig. 1). Approximate estimation of their sedimentation coefficients in the density gradient sedimentation diagram gave S numbers of 17 and 22. It was quite likely, therefore, that these RNAs represented nascent forms of two rRNA components. Nucleotide composition and sedimentation characteristics of rRNA formed during longer period of labelling (10-15 min.) were identical with those of the typical 16S and 23S rRNA.

EARLY COMPONENTS IN RIBOSOME FORMATION

Time course was then followed for the incorporation of labelled precursors of RNA [(^{3}H) adenosine or (8-^{14}C) adenine] into various components resolved by sedimentation analysis of bacterial extracts prepared from early "shift-up" cells at a magnesium concentration of 0·1 m moles[7]. At 15 sec. of pulse-labelling, two discrete radioactive components of 18S and 22S were observed (Fig. 2A). Beside these earliest components, three additional heavier components of 25S, 29S and 32S appeared at 1 min. of labelling (Fig. 2B). Some radioactive components smaller than 16S were always found in short-time labelling experiments. They are probably breakdown products of larger components and some mRNA. At 15 min. of labelling, the bulk of the radioactivity was found in 30S and 50S ribosomal subunits and also some in 4S region. The earliest components apparently represented the free nascent rRNA mentioned above. Obviously, transfer RNA (tRNA) synthesis lagged behind rRNA synthesis. This point agrees with recent findings of Midgley[8].

EFFECTS OF CHLORAMPHENICOL AND 5-FLUOROURACIL

Chloramphenicol (CM) is known to cause inhibition of ribosome formation and accumulation of smaller particles of 18S and 25S[9]. The same was true with our experimental conditions. Incubation of "shift-up" cells for 15 min. in the presence of CM (200 μg./ml.) resulted in the appearance at 18S and 25S regions of radioactive peaks and corresponding peaks of absorbancy at 260 mμ, with little radioactivity at higher S regions (Fig. 3A). On removing CM and the labelled precursor, the radioactivity was quantitatively transferred during a further incubation of cells for 60 min. to the normal 30S and 50S subunits[7] (Fig. 3B). As mentioned above, components similar to the CM particles were detected among early components revealed with normal "shift-up" cells.

It is known for some time that 5-fluorouracil (FU) affects the course of ribosome formation[10,11]. We, therefore, conducted a series of experiments to learn the effects of FU in our system. When "shift-up" cells were exposed to FU (20 μg./ml.), 30S region was labelled during the contact with (^{14}C) adenine for 10 min. The 50S region was not labelled (Fig. 4). The same

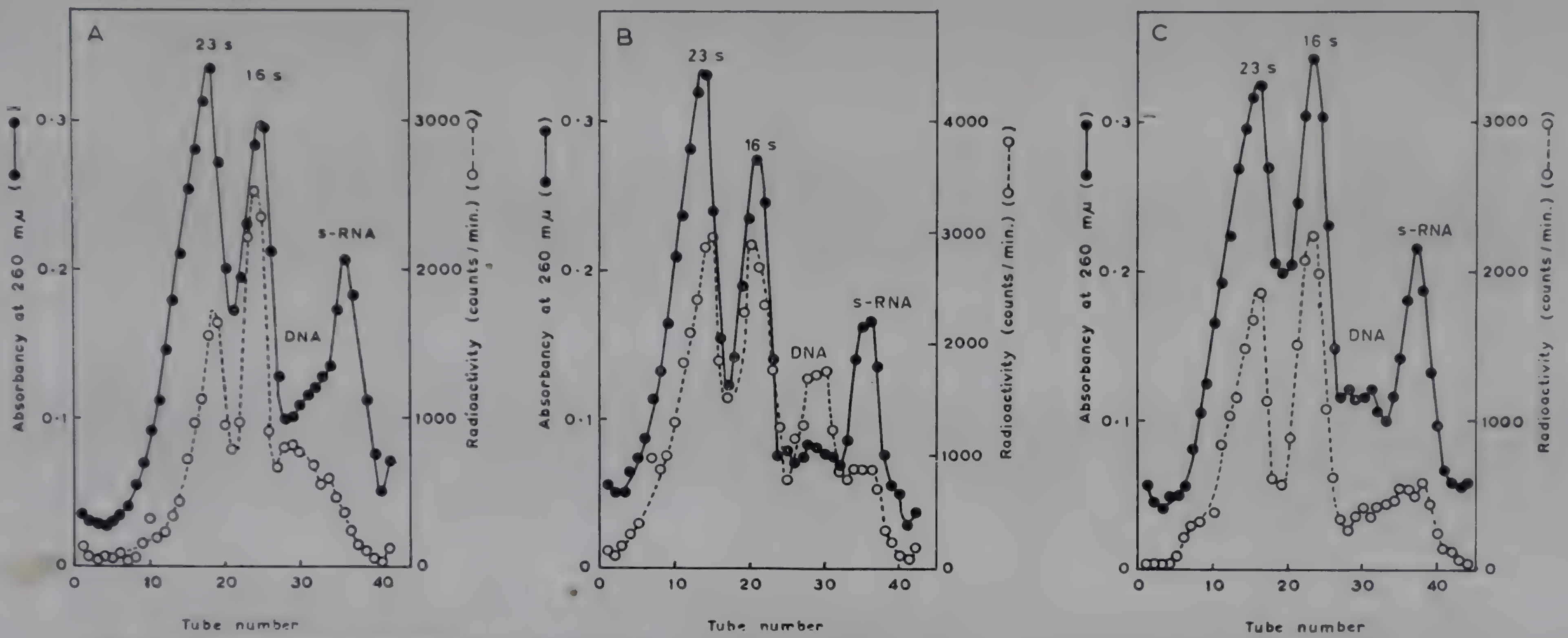


FIG. 1—SEDIMENTATION ANALYSES OF RNA LABELLED WITH (^{3}H) URIDINE FOR (A) 20 SEC., (B) 1 MIN. AND (C) 10 MIN., STARTING 6 MIN. AFTER THE TRANSFER OF BACTERIA TO THE "SHIFT-UP" CULTURE MEDIUM (From ref. 4)

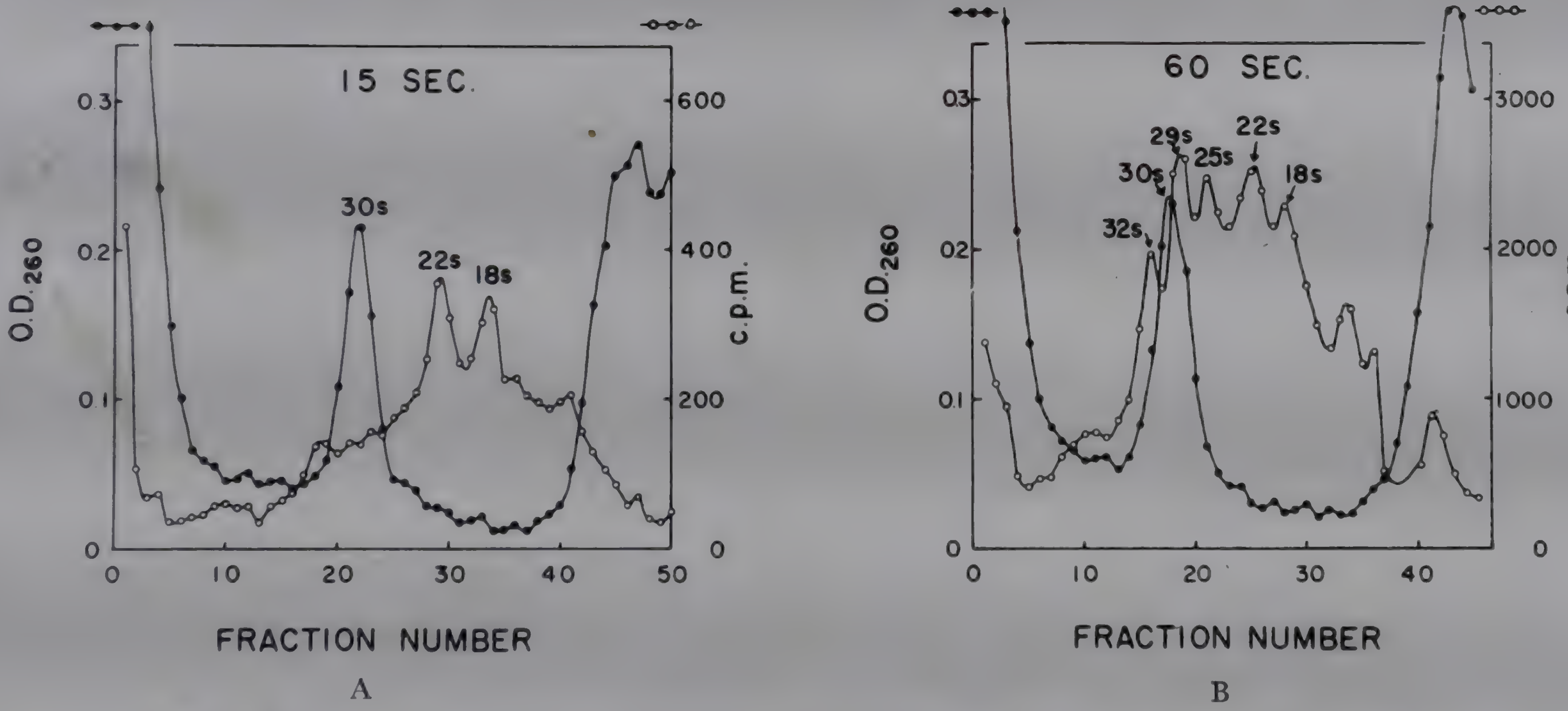


FIG. 2—SEDIMENTATION ANALYSES OF CRUDE EXTRACTS FROM "SHIFT-UP" CELLS PULSE-LABELLED WITH (^{3}H) ADENOSINE FOR (A) 15 SEC., AND (B) 1 MIN. (From ref. 7)

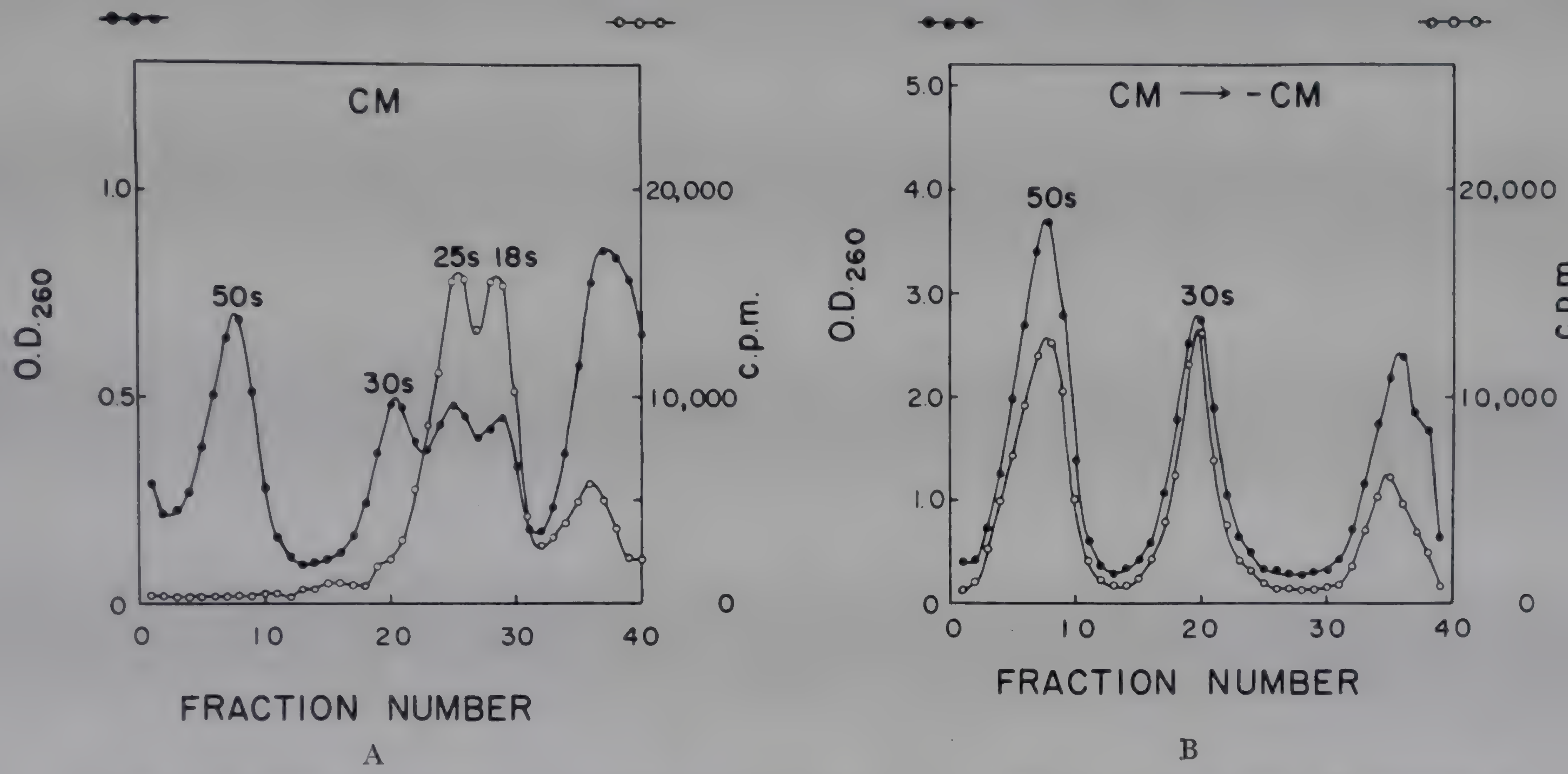


FIG. 3—SEDIMENTATION ANALYSES OF A CRUDE EXTRACT FROM "SHIFT-UP" CELLS LABELLED FOR 15 MIN. WITH (^{3}H) ADENOSINE IN THE PRESENCE OF 200 μg./ml. OF CM : (A) appearance at 18 and 25S regions of radioactive peaks and corresponding peaks of absorbancy at 260 mμ, with little radioactivity at higher S regions and (B) on removing CM and the labelled precursor, the radioactivity is quantitatively transferred during a further incubation of cells for 60 min. to the normal 30S and 50S subunits [From ref. 7]

pattern of labelling was obtained with longer exposure to the isotopic precursors or even by using (^{3}H) fluorouracil as the label. Accumulation of components around 30S was also noted from the absorbancy curve. So far the results agreed with those of the previous observations by Aronson[10] and Gros *et al.*[11] using steadily growing cells, but discrepancies emerged in the following. Upon further incubation of FU-treated cells without FU and the isotopic precursor, the radioactivity became evenly distributed between normal 50S and 30S subunits. Labelled RNA obtained from FU-treated cells gave two peaks of 17S and 22-23S in sedimentation diagram. They would correspond to the nascent forms of rRNA as observed by pulse-labelling the "shift-up" cells. Therefore, particles sedimenting around 30S region should contain, unlike normal 30S ribosomal subunit, two RNA components which would represent "premature" state of normal rRNA components. Actually, a closer analysis of labelled particles in the presence of FU revealed two peaks of 28S and 32S (Fig. 5B). They may be called conveniently the FU particles. They appear to correspond to some of the early particles observed by pulse-labelling the "shift-up" cells. Moreover, label of the FU particles was rendered acid-soluble by ribonuclease (1 μg./ml.) at room temperature, whereas normal 30S subunit was little affected

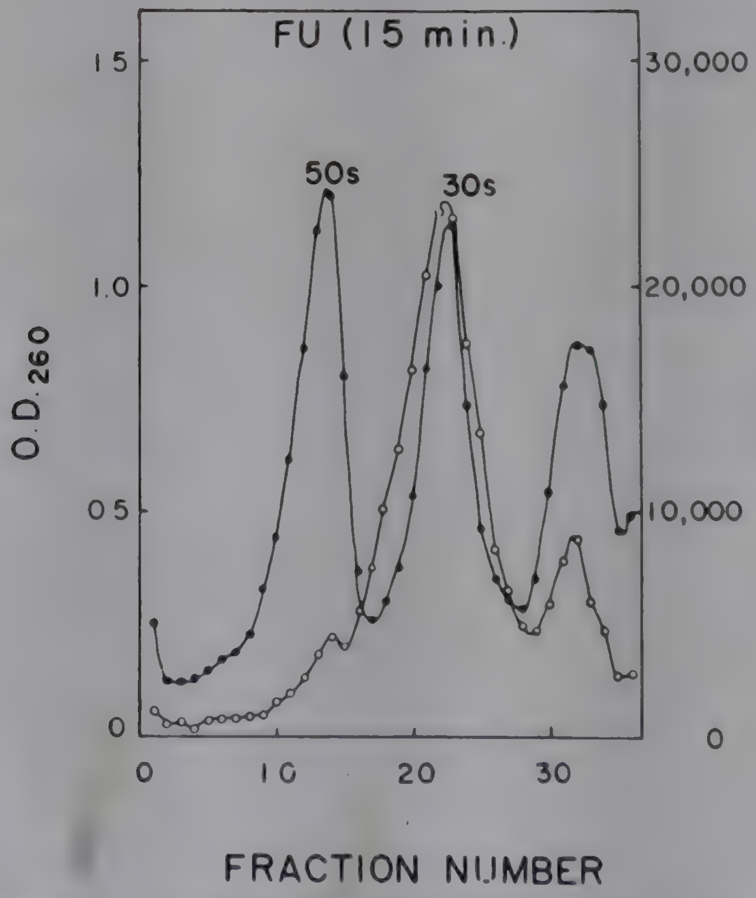


Fig. 4—Sedimentation analyses of a crude extract obtained from "shift-up" cells labelled with (^{14}C) adenine for 10 min. in the presence of 20 μg./ml. of FU [From ref. 7]

under the same conditions. It can thus be concluded that FU particles around 30S are not mature 30S subunits but represent immature particles which are accumulated in the presence of FU. Also, the pattern of labelling of early components was not affected by FU[7].

FU thus seems to effect a reversible blockade to the maturation step of ribosomes beyond the stage represented by "early components".

In order to test the relationship between CM particles and FU particles, "shift-up" cells labelled with (^{3}H) adenosine for 15 min. in the presence of both inhibitors were freed of these ingredients in the medium and further incubated for 40 min. with FU. It was thus demonstrated that the labelled CM particles were converted into FU particles in the absence of CM (Fig. 5A, B). It was also confirmed, in the same series of experiments, that the label found in FU particles was quantitatively transferred to normal 30S and 50S ribosomal subunits upon removal of FU from the medium (Fig. 5C). In such an experiment, a 38S component (Fig. 5C) and sometimes also a 43S component tended to appear. They may represent further intermediates of 50S subunit beyond the FU particles.

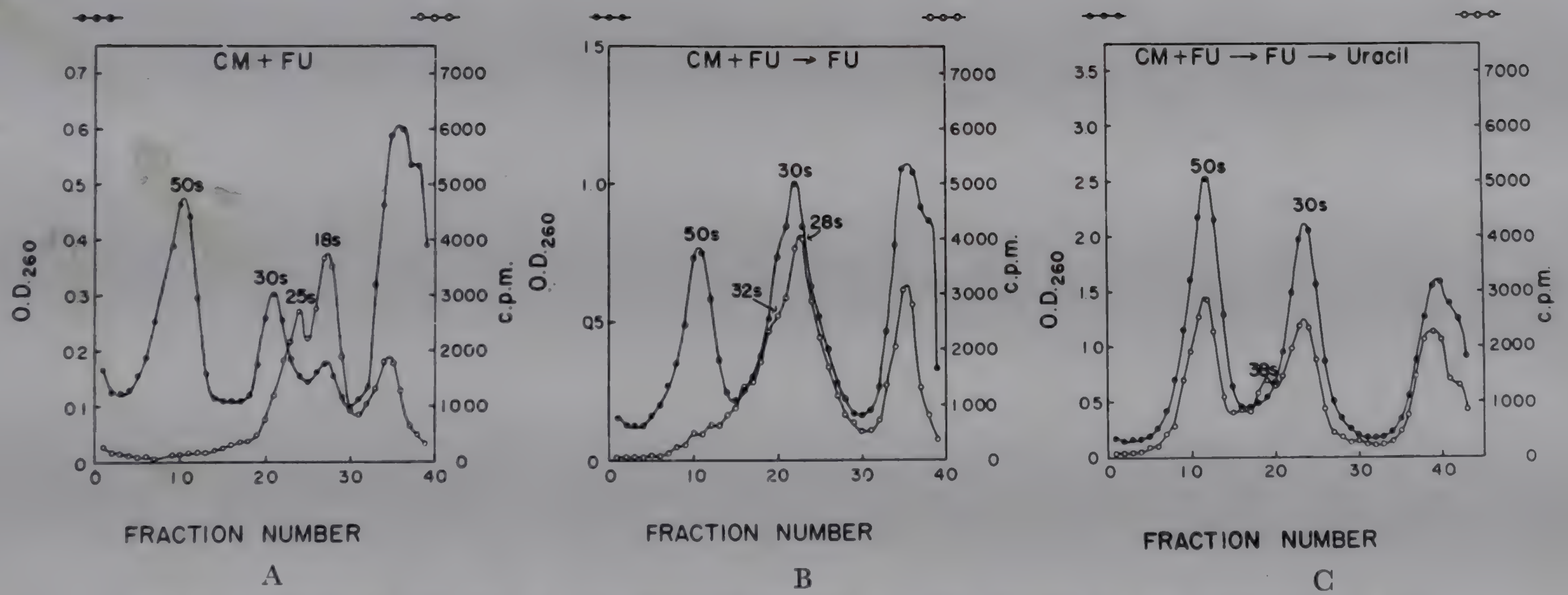
Differential Inhibition of Ribosome Formation with Ultraviolet Light

It was demonstrated recently by Rörsch *et al.*[12] and by ourselves that the formation of 50S ribosomal subunit was more sensitive to ultraviolet irradiation than that of 30S subunit. Correspondingly, at the rRNA level, synthesis of 23S component was more readily blocked by ultraviolet irradiation than that of 16S component[5] (Fig. 6). Chromatographic analysis of methylated serum albumin column revealed that some synthesis of mRNA and tRNA could be observed even at the dose which completely suppressed the synthesis of rRNA. The use of "shift-up" culture would obviate the complication caused by mRNA.

Sedimentation analyses of ribosome formation were conducted using "shift-up" cells which were labelled with (^{3}H) adenosine following the exposure to varying doses of ultraviolet light. Conditions are available under which the formation of 50S subunits was practically nil while there was a substantial synthesis of 30S subunits. The dose of ultraviolet light effecting this with "shift-up" cells (1300 erg/mm.^{2}) was comparable to that giving the same type of results with normally growing cells. This system can now be employed for identifying intermediates of 30S subunits and 50S subunits separately at various stages of ribosome formation. Experiments along this line are now in progress.

Ribosome Formation Following Magnesium Starvation

McCarthy[13] reported that, when *Esch. coli* cells were subjected to



A B C

FIG. 5—SEDIMENTATION ANALYSES OF CRUDE EXTRACTS PREPARED (A) AFTER EXPOSURE TO (^{3}H) ADENOSINE FOR 15 MIN. IN THE PRESENCE OF CM AND FU, (B) AFTER SUBSEQUENT INCUBATION FOR 40 MIN. IN A SHIFT-UP MEDIUM WITH FU BUT WITHOUT CM AND (^{3}H) ADENOSINE, AND (C) AFTER FURTHER INCUBATION FOR 60 MIN. WITHOUT BOTH FU AND CM IN THE SAME MEDIUM [From ref. 7]

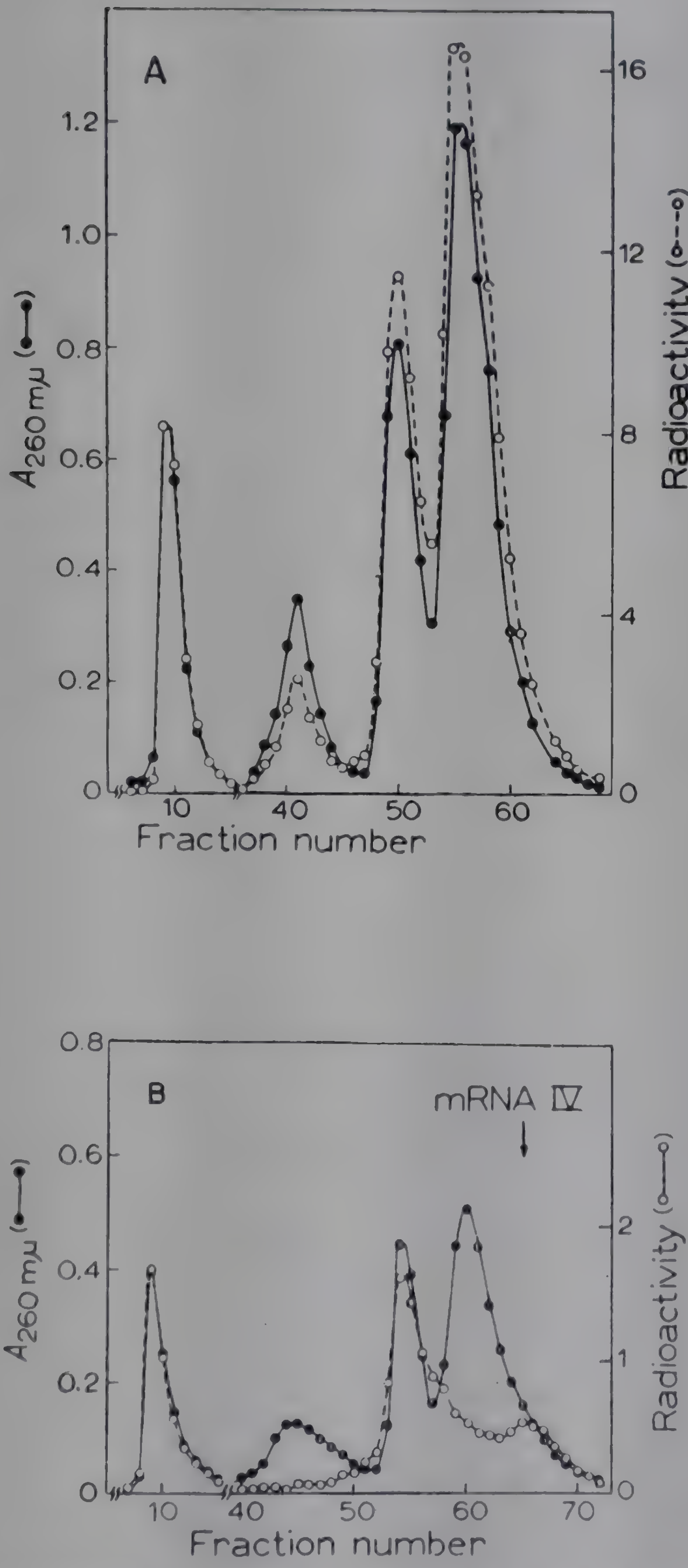


Fig. 6—Chromatographic analyses of RNA labelled for 30 min. with inorganic ^{32}P obtained from (A) control cells and (B) cells irradiated with about 1400 erg/mm². ultraviolet light from a germicidal lamp: Cells were cultured in Tris-glucose-Casamino acid medium [From ref. 5]

magnesium starvation, the cells were depleted of preformed ribosomes. We now found that, upon replenishment of magnesium ions to such cells, the synthesis of what appeared to be the "premature" rRNA (17S and 22S) was immediately resumed, while the protein synthesis was suppressed for a period of more than 90 min. The "premature" RNA was incorporated into particles of 18S and 25S which appeared to correspond to CM particles (Suzuki, H. and Hayashi, Y., unpublished data). The situation, therefore, paralleled that prevailing when bacteria were treated with CM. The advantage of using such a system lies in the fact that cells are practically devoid of mature ribosomes so that a bulk isolation of early particles and of "premature" rRNA is feasible.

Separate isolation of 17S and 22S RNA from magnesium-replenished cells was accomplished and their nucleotide composition and hyperchromicity by heating were examined (Suzuki, H. and Hayashi, Y., unpublished data). The 17S and 22S components showed very similar nucleotide compositions closely resembling that of rRNA. There emerged, however, a definite difference in the secondary structure between "premature" RNA and authentic rRNA. Both 17S and 22S RNA exhibited a significantly lower hyperchromicity upon heating than 16S and 23S rRNA. "Premature" RNA detected by pulse-labelling of "shift-up" cells formed rapidly sedimenting aggregate much more readily than rRNA at higher magnesium concentration (up to 10 m moles), which also suggested a difference in the secondary structure.

Using RNA isolated from magnesium-replenished cells, we also carried out sedimentation analysis of "premature" RNA components mixed with a minute amount of heavily labelled normal rRNA as reference material. We could thus confirm that the slight difference in sedimentation coefficients noted above between the "premature" and mature rRNA (17S and 22S *versus* 16S and 23S) was a reality and not occasioned by an aggregation of smaller components with some of the rRNA molecules in former experiments, in which "premature" RNA was labelled on the background of normal unlabelled rRNA. Such a difference may well be related to the noted difference in the secondary structure of these RNAs.

INFORMATION CONTENT OF RIBOSOMAL RNA

Questions concerning the origin of ribosomal protein are pertinent to the problem of ribosome formation. rRNA itself may have an information for amino acid sequence of ribosomal or even some other proteins. This supposition is not very unlikely in view of the high degree of degeneracy in the genetic code. The present knowledge in molecular biology points to the necessity of association of template RNA with the ribosome for the synthesis of a polypeptide[14]. We, therefore, first examined the ability of combination of rRNA prepared from mature ribosomes with 30S, 50S, 70S and 100S ribosomes under the conditions under which natural messenger RNA[6] or some synthetic polyribonucleotides[15] attaches to ribosome (5-10 mM Mg^{2+}). The combination was followed by sucrose density gradient centrifugation. In no case did any significant interaction occur between rRNA and ribosomes. It was then found that a small amount of spermidine (5 m moles), which had been shown to enhance the poly-U-dependent polyphenylalanine formation in cell-free systems of bacteria or yeast[16,17], caused a quantitative association of rRNA to 70S ribosome in a proportion of at least one 23S or two 16S rRNA molecules to one ribosome. Under these conditions, 30S particles showed a much greater affinity for rRNA than 50S particles. This observation is in accord with that of Takanami and Okamoto[15,18] who observed the predominant combination of various polynucleotides (excluding, however, rRNA) with 30S subunits. No heavy ribosomes were formed in the presence of spermidine.

Although spermidine greatly stimulated the formation of rRNA-ribosome complex, it failed to give rise to a significant amino acid [(^{14}C)-leucine] incorporation into proteins in systems similar to those of Singer *et al.*[19] and Nierenberg and Matthaei[20], and Takanami and Okamoto[15]. However, poly-U-dependent polyphenylalanine formation was stimulated up to 60-fold by spermidine. Moreover, the presence of an excess amount of rRNA added in the system did not affect the extent of polyphenylalanine formation (Table 2). These results indicate that the combination of polynucleotide with ribosome is necessary but not sufficient for the synthesis of protein. The results also suggest that the site on a ribosome, or else the particular species of ribosomes, specialized for attachment of rRNA may be different from that for interaction with poly-U.

The finished rRNA used in the above experiments undoubtedly has ordered structures to a considerable extent which might interfere with the code reading as suggested by Singer *et al.*[19] and by Takanami and Okamoto[5,18]. We, therefore, thought that 17S and 22S "premature" rRNA's which supposedly have a less ordered structure, might possess the ability to stimulate amino acid incorporation into proteins. As massive isolation of our earliest components (free "premature rRNA") is difficult at the present time, we isolated RNA from cells of a "shift-up" culture in the presence of CM, because rRNA in CM particles was shown to be still in the "premature" state. CM was added at the beginning of shift-up culture, and the incubation was continued for 12 min. Nucleic acids were then isolated as described previously[6]. Isolated nucleic acids were then treated with DNAse and centrifuged in a sucrose density gradient for 16 hr at 25,000 r.p.m. The following fractions were collected after centrifuging: (I) 23S (>20S), (II) 16S (15-20S), (III) 14S (7-15S) and (IV) 4S (<7S). These fractions were concentrated with ethanol precipitation and tested for the stimulation of (^{14}C) leucine incorporation into proteins. It was found that 42·3, 38·4, 19·4, and 0 % of the activity for amino acid incorporation

TABLE 2—Effects of Ribosomal RNA and Poly U on Amino Acid Incorporation into Hot Trichloroacetic Acid-insoluble Material in 30 S Fraction of *Esch. coli**

Type of added polynucleotide	Spermidine† 0·005 *M*	Labelled amino acid used	Counts per min. per tube in hot trichloroacetic acid-insoluble fraction
–	–	(^{14}C) leucine	1,395
rRNA (0·8)‡	–	do	1,323
rRNA (2·4)‡	–	do	1,485
rRNA (4·8)‡	–	do	1,475
–	+	do	1,529
rRNA (0·8)‡	+	do	1,456
rRNA (2·4)‡	+	do	1,619
rRNA (4·8)‡	+	do	1,646
–	–	(^{14}C) phenylalanine	205
poly-U (0·6)‡	–	do	351
–	+	do	528
poly-U (0·6)‡	+	do	9,982
	+	do	85
rRNA (12·6)‡	+	do	86
poly-U (0·3)‡	+	do	10,899
poly-U (0·3)‡ +rRNA (12·6)‡	+	do	15,104

*Otaka, E., Osawa, S. & Sibatani, A. (unpublished data).

†In case of spermidine addition, Mg^{2+} concentration was reduced to 0·005 *M*.

‡Total absorbancy (1 cm. optical path) at 260 mμ added to the reaction mixture.

was present in Fractions I, II, III and IV, respectively. More precise fractionation and characterization of the active RNA component(s) is now in progress.

Conclusion

From the experimental results outlined above, we can now conclude that, following the first appearance of the "premature" RNAs, the course of ribosome formation may proceed via several intermediary stages with increasing sedimentation coefficients. The increase in particle size probably means increasing amounts of proteins incorporated into the particles, whereas their RNA components have a completed primary structure from the very start of ribosome formation.

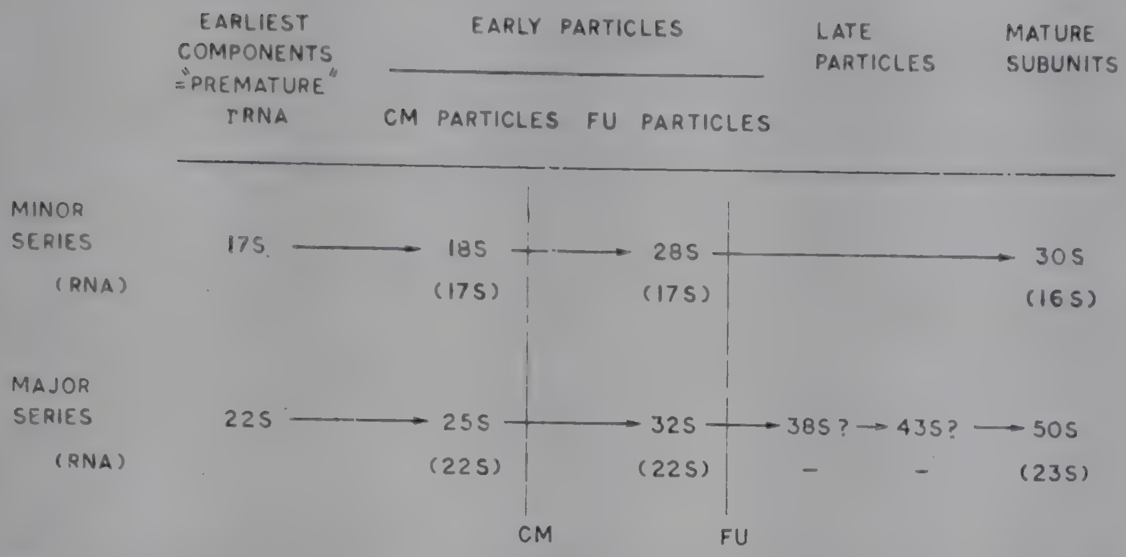

EARLIEST COMPONENTS ="PREMATURE" rRNA
EARLY PARTICLES
CM PARTICLES
FU PARTICLES
LATE PARTICLES
MATURE SUBUNITS
MINOR SERIES
(RNA)
17S
18S
(17S)
28S
(17S)
30S
(16S)
MAJOR SERIES
(RNA)
22S
25S
(22S)
32S
(22S)
38S?
43S?
–
–
50S
(23S)
CM
FU

FIG. 7—A TENTATIVE SCHEME FOR THE FORMATION OF RIBOSOMAL SUBUNITS IN *Esch. coli*

Thus, our tentative scheme of ribosome formation is at variance with the hypothesis advanced by Britten *et al.*[14] In their eosome-neosome concept, a 14S RNA (eosome) grows into mature and larger rRNA and simultaneously picks up increasing amounts of proteins to pass a neosome stage, and finally to complete the maturation into ribosome subunits of 30S and 50S.

In our scheme, two ribosome subunits are formed separately from the start. This idea is in consonance with the notion that 23S and 16S rRNA components are probably synthesized on separate cistrons. From what has been learned so far, the two series of subunit formation may be formulated as in Fig. 7.

The origin of ribosomal protein is still entirely obscure. Our experiments described in the last section suggest that the "premature" rRNA may have some information for protein synthesis. The possibility does exist that rRNA thus directs the synthesis of ribosomal protein.

ACKNOWLEDGEMENT

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The Molecular Basis of Crossing-over

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An adequate theory of crossing-over has to relate three different sets of facts to each other, the results of genetic analysis, the microscopic observations on the chromosomes and the molecular structure and properties of the genetic material. Formal genetics deals with the geometry of the subject, i.e. how the exchanges are distributed along the length of a chromatid or between the chromatids of a tetrad[1,2]. At the cytological level these results are interpreted by the models of classical genetics[3]. With the advent of fine-structure genetic analysis and the knowledge that DNA is the repository of genetic information, the realization has grown that the cytologically observable meiotic chromosomes are orders of magnitude removed from the level at which elementary genetic events occur. There have consequently been many attempts to construct models of a new kind which seek to interpret crossing-over at the molecular level[4–6].

At present we know little about the molecular organization of the chromosomes in higher organisms. A number of interesting molecular or quasi-molecular structures have been proposed, some of which possess considerable versatility and elegance[6–9]. Most of these models are, however, tailored to fit the known facts of genetic exchange and in using them to unravel the molecular basis of crossing-over one has to guard against circular argument. Fortunately the situation is simpler in the case of bacteria and bacteriophages where there are good reasons to believe that the chromosome consists of a single continuous piece of DNA[10]. The recent autoradiographic experiments of Cairns provide direct support for this view[11,12]. The following discussion of crossing-over is based mainly on evidence obtained from microorganisms with the underlying assumption that crossing-over occurs within the DNA molecule. Needless to say this may not always be so. Information on crossing-over comes from different organisms and is based on diverse genetic systems such as meiotic or mitotic crossing-over, transformation, transduction or conjugation. There is the distinct possibility that we may be dealing with more than one mechanism. Nevertheless, the consequences of crossing-over in different genetic systems are very similar; in particular the fine-structure maps derived from these diverse systems resemble each other so closely that one is encouraged to look for a common basic mechanism. In the present article we consider a few selected aspects of genetic recombination restricting ourselves to the following questions: (1) Does crossing-over involve breakage and reunion of pre-existing DNA molecules ? (2) Is crossing-over separable from DNA replication; how are the two related ? (3) How do homologous segments of DNA interact specifically to give rise to recombinant DNA ? A number of recent experiments provide information relevant to the first two questions; as for the nature of genetic pairing, at present one can only speculate. We do so in the hope that some of the conjectures can be tested experimentally.

Some novel features of genetic recombination

The study of recombination in microorganisms has revealed some features of crossing-over which were not evident in classical studies. Three of these novel phenomena, non-reciprocal exchange, negative interference and polarization, appear to be widespread. They become evident at a level of analysis which is close to atomic dimensions and seem to offer clues to physical mechanisms[5,13].

In 1953, Lindegren described unusual tetrads in yeast with allele ratios of 3:1 instead of the usual 2:2. This was called gene-conversion and was believed to be a kind of directed mutation[14]. Subsequent work amply confirmed the occurrence of 3:1 segregation in tetrads[15]. If we consider a double-heterozygote for two-linked markers one of which undergoes conversion, the tetrad will contain a recombinant strand, the reciprocal of which is missing. Gene-conversion thus involves a non-reciprocal exchange of information between chromosomes. Several lines of evidence indicate that gene-conversion is itself a recombinational rather than a mutational event and is in some way connected with crossing-over[13,16–18]. Since the input and output ratios of markers in conversion type tetrads are not the same, this kind of recombination cannot be explained by any simple model based on a breakage and reunion of four parental strands. In fact non-reciprocal exchange at present constitutes the chief argument in favour of recombination by copy choice.

High negative interference is an observation of a purely statistical nature. When crossing-over between closely linked markers is studied, multiple exchanges over short segments of the chromosomes show a strong positive correlation. This has been interpreted to mean that the exchanges along a chromosome are confined to a small number of randomly distributed regions, the so-called effective pairing segments[13] or switch areas[19]. On the basis of some subsidiary assumptions the parameters characterizing the pairing segments, such as their mean length or the mean frequency of exchange per segment, can be calculated. The physical meaning of high negative interference is not quite clear. Indeed the current interpretation is open to some doubt[5]. The phenomenon itself is, however, quite striking.

Polarization of exchanges was first convincingly demonstrated by Rizet and his colleagues[18]. Working with a set of ascospore colour mutants of *Ascobolus immersus* they found that recombination between these was invariably non-reciprocal, involving the conversion of one of the alleles in the cross. The mutations could be arranged in a unique linear map on the basis of the conversion frequencies. The striking fact is that in a cross between any two it is always the allele towards one side which is converted. For instance, if the order is 1, 2, 3 . . . and in the cross 2×3, the allele on the left, that is 2, is converted, then in the cross 1×2, 1 would be converted and so on. A particular region of the chromosome where this rule holds is defined as a polaron and is explained by assuming that DNA replication, which constitutes an essential condition for exchange, proceeds from one end of the polaron to the other. We have looked at the problem of polarization in a different way[20,21]. In crosses between allelic mutants of *Aspergillus nidulans* requiring *p*-aminobenzoic acid we compared the localized negative interference on the two sides of the interval of selection. It turned out that there was a marked asymmetry between the proximal and the distal intervals indicating polarization of recombinational events. Similar results have been obtained by Dr Noreen Murray with *Neurospora crassa*[22]. The precise interpretation of these results need not concern us for the present. It is clear, however, that some sort of polarization exists. There is what might be called a 'non-conservation of parity' between the 'right' and the 'left' sides with respect to crossing-over.

Transmission of parental DNA to recombinant progeny

Two different kinds of physical mechanisms have been considered to cause genetic crossing-over. According to the first scheme the linear bonds of homologous chromosomes break at corresponding points and the broken ends rejoin in such a way that equivalent segments are interchanged between the two chromosomes. This is called breakage and reunion. According to the second scheme the parental strands themselves remain intact. When a new chromosome is synthesized it may use both parental strands as templates resulting in a chromosome which combines genetic information from two different chromosomes. The two hypotheses are not exclusive of each other but they make different predictions about the distribution of the parental DNA among the progeny. In breakage and reunion the DNA of the parental chromosome is transmitted to the recombinant. In copy choice the recombinant chromosome is synthesized *de novo* and does not contain atoms of the parental DNA.

Meselson and Weigle[23] labelled the DNA of phage lambda with ^{13}C and ^{15}N and crossed it with an unlabelled strain differing in the genetic markers *c* and *mi*. When the progeny phages ensuing from this cross were

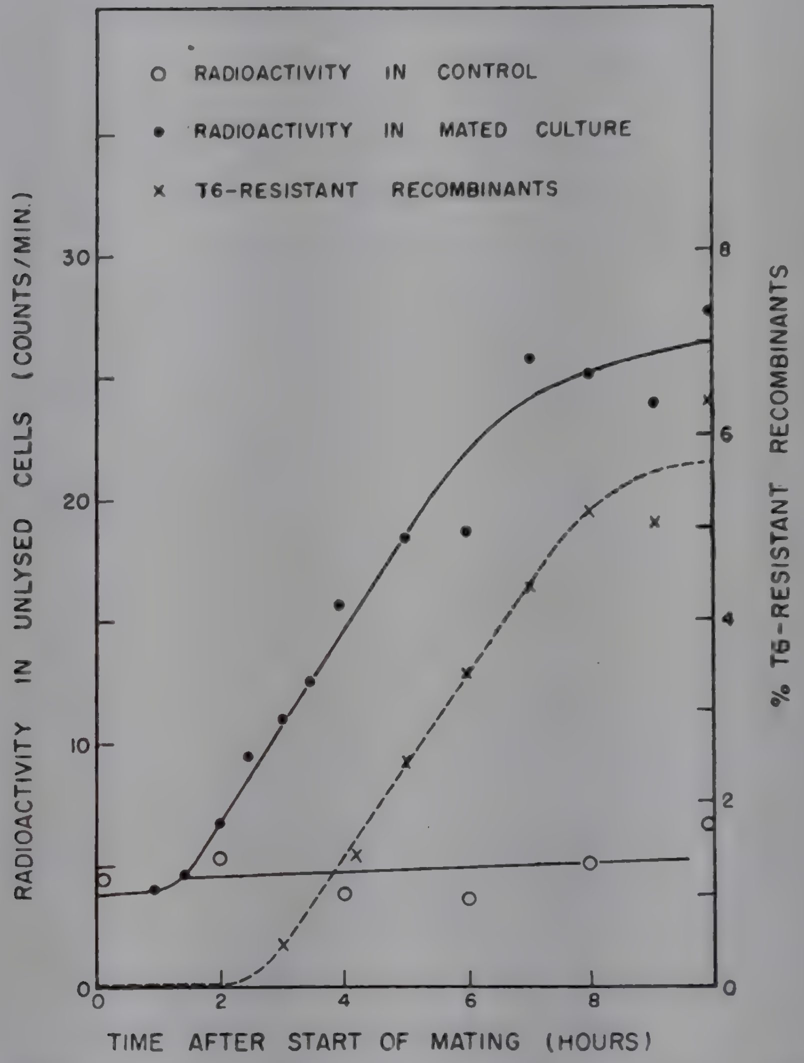

FIG. 1—TRANSMISSION OF ^{3}H-THYMIDINE LABELLED DNA FROM F^- TO T6-RESISTANT RECOMBINANTS: Mating: Hfr $T6^rS^s \times F^-T6^sS^r$(^{3}H-labelled). Aliquots of mated cells were treated with phage T6 and DNAase. The unlysed cells were separated by filtration and examined for radioactivity. The radioactivity in the unlysed cells on the filter paper rises in parallel with the titre of $T6^rS^r$ recombinants

banded in a caesium chloride density gradient it could be shown that the genetic recombinants contained labelled DNA. In other words, recombination involved the breakage of the parental DNA. Meselson and Weigle[23] were able to draw a number of conclusions, the most important being (1) that breaks leading to recombination must involve both strands of DNA; and (2) recombination does not need DNA-replication. Kellenberger, Zichichi and Weigle[24] have used a slightly different experimental system to arrive at similar conclusions.

We have carried out an analogous experiment with the bacterium *Escherichia coli*[25]. A streptomycin-resistant F^- was labelled with tritiated thymidine and mated to an Hfr which was resistant to phage T6. The mated cells were allowed to grow and at regular intervals aliquots of cells were lysed with a heavy dose of phage T6. The unlysed streptomycin-resistant T6-resistant recombinants were separated by filtration and the radioactivity in their DNA was measured. These cells contained discrete amounts of the parental DNA. The results of a typical experiment are shown in Fig. 1. After eight hours of growth, about 6 % of the F^- are T6-resistant. Since these contain 4-5 % of the radioactivity in the F^- parent it would appear that, as in the phage lambda, both strands of the parental DNA are transmitted to the recombinants.

In our experiments, as in those of Meselson and Weigle, and Kellenberger, Zichichi and Weigle, only one of the parents contained labelled DNA. Strictly speaking, these experiments demonstrate the association of 'old' DNA with 'new' genetic information but not reunion of pre-existing DNA fragments. Bodmer and Ganesan[26] working with the transformation system of *Bacillus subtilis* labelled the DNA of both parents differentially and demonstrated the association of the two parental labels in the recombinant DNA. One may, therefore, conclude that at least a part of crossing-over in bacteria and bacteriophages involves breakage and reunion of pre-existing DNA molecules. The occurrence of recombination by breakage does not exclude a mechanism based on a copy choice. It needs to be emphasized though that so far the evidence in favour of such a mechanism is only indirect and inferential.

Recombination and DNA replication

For a mechanism based on copy choice, crossing-over is an integral part of DNA replication. This is not so for breakage and reunion. The question naturally arises: is crossing-over inseparably connected with replication or are the two processes dissociable? The experiments of Meselson and Weigle already described indicate that the lambda chromosome may recombine without undergoing replication. Fox used ^{32}P-labelled DNA to transform *Pneumococci*[27,28]. The loss of transforming activity as a result of ^{32}P decay in the donor DNA remained unchanged in the DNA reisolated from the recipient after integration. Using the appearance of linkage between the markers of the donor and the recipient as a measure of crossing-over, Fox was able to show that recombination is completed in the absence of any DNA synthesis. These experiments show that in pneumococcal transformation, crossing-over is independent of replication. Other possible connections between replication and recombination, however, remain to be explored. These may be visualized in several ways.

1. There may exist a special kind of recombination such as copy choice which is an integral aspect of DNA replication.

2. Some pre-requisites may be common to replication and crossing-over. The two may thus be correlated though not interdependent. For instance, the unravelling of a tightly-packed DNA during replication may expose it to crossing-over. There may be an apparent connection due to reasons having nothing to do with the mechanism of crossing-over. The transfer of the Hfr chromosome to the F^-, for example, appears to require the initiation of DNA replication (R. H. Pritchard, personal communication).

A molecular model for genetic pairing

By pairing we denote an association between homologous chromosomes which permits crossing-over. If genetic information is to be conserved, exchanges must occur at precisely homologous points along the length of the interacting chromosomes. This is best visualized under conditions when the chromosomes are synapsed. We have already discussed the evidence which indicates that crossing-over between DNA molecules must involve both chains. The question arises how do segments of double-stranded DNA recognize each other. Since the genetic information resides in the sequence of bases it is reasonable to assume that mutual recognition between DNA molecules depends on the hydrogen bonding of the bases. The following model is an attempt to account for specific pairing of homologous DNA segments.

Consider a cross-section of the DNA double helix at right angle to its axis. Since each inward-pointing base occupies a quadrant, there is room in the helix for four bases. In other words, two double-helices can be wound around each other without much distortion of the Watson-Crick structure. In a normal double helix the paired bases occupy adjacent quadrants. If the two helices are rotated by 90° in opposite directions the bases will again occupy adjacent quadrants with hydrogen bonding positions exposed. If a second double helix with an identical sequence of bases is also transformed by rotating the chains in opposite directions the two helices can be combined specifically in a quadruple helix (Fig. 2). Note that the rotation of the chains in one of the double-helices will have to be such that the backbones slip over each other. The formation of such quadruple helices has been proposed in connection with DNA replication[29–31]. We make use of the suggestion to account for specific pairing between DNA molecules without any implications as regards the replicative form of DNA. Since

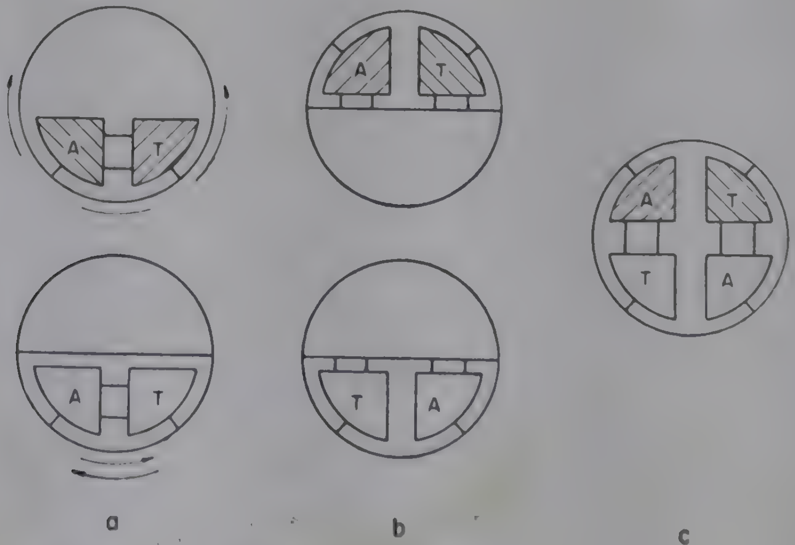

FIG. 2—CROSS-SECTION OF TWO DNA DOUBLE-HELICES SHOWING ROTATION OF THE BACKBONES LEADING TO SPECIFIC PAIRING

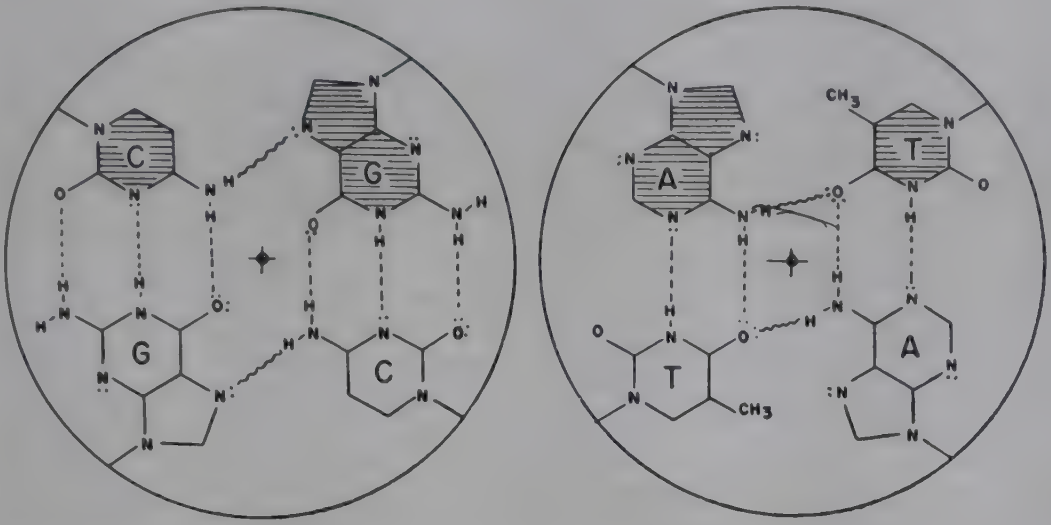

Fig. 3—Cross-section of a quadruple helix showing hydrogen bonding between the paired duplexes (after Löwdin[31])

each turn of the helix has ten base pairs no more than one or a few turns of the quadruple helix will be needed to impart sufficient specificity to pairing. It has been pointed out by Lowdin[31] that two more hydrogen bonds could be formed in the quadruple helix so that the quadruple helix might have additional stability (Fig. 3). The helices will have the dimensions of the Watson-Crick structure except near the ends of the quadruple region where the chains will undergo some distortion.

A simple scheme leading to specific pairing is envisaged in Fig. 4. The chromosomes are assumed to undergo localized unwinding and denaturation over short regions. Two such opened-out regions may come together and the four strands may rewind into a quadruple helix. The possibility of localized opening and rewinding of the double helix derives circumstantial support from the work of Doty and his colleagues[32,33] on reversible denaturation of DNA.

If a substantial portion of the DNA existed in the four-stranded form, this would be recognized by physical methods. The existence of a four-stranded state in connection with DNA replication has generally been discounted (Cavalieri and Rosenberg)[34,35]. For the purpose of pairing, however, only a small fraction of DNA need form quadruple helices. This may not be detected by the usual techniques.

The following features of the proposed scheme of pairing are of interest because of their genetic consequences: (1) Within the paired region the homologous segments of DNA are aligned recon by recon. This provides the most favourable geometry for exchange of linear bonds between the backbones. (2) If crossing-over involved only one strand of the parental duplex the paired double helices will not be able to separate without breaking all hydrogen bonds between the point of exchange and the end of the chains. On the other hand, if both strands underwent breakage and reunion, only the hydrogen bonds in the paired region need break to allow the

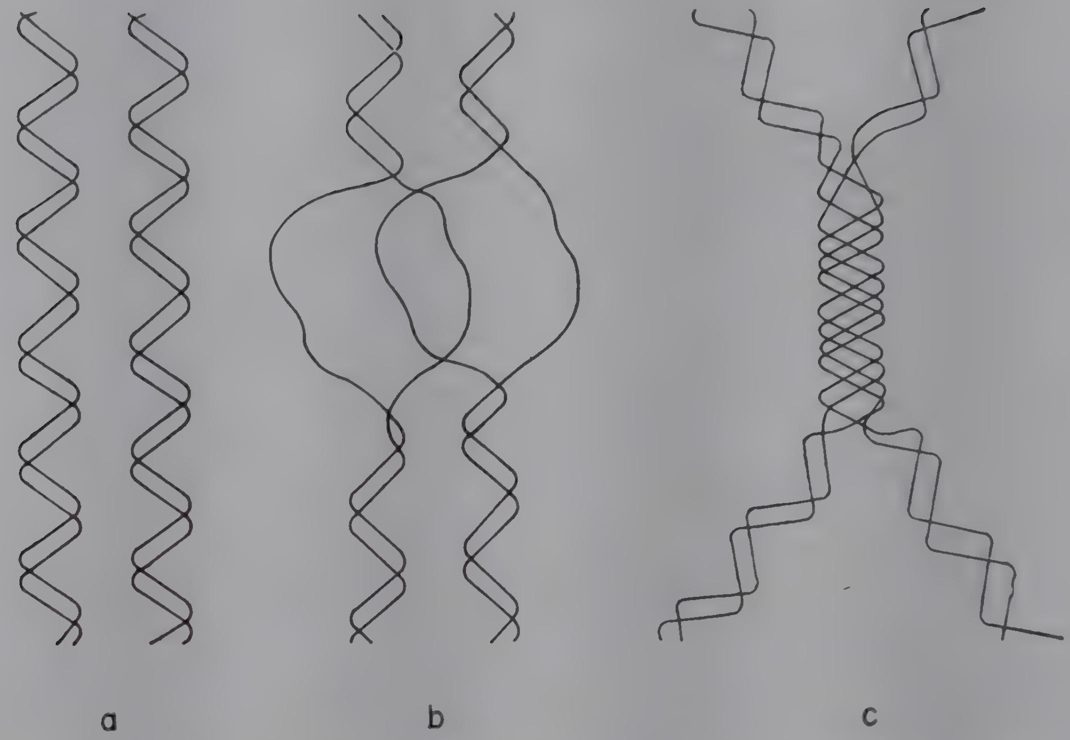

FIG. 4—PAIRING BY LOCALIZED DENATURATION: (*a*) parental duplexes; (*b*) localized unwinding; (*c*) quadruple helix formation

chromosomes to separate. This imposes a restriction favouring the crossing-over of both chains. (3) The quadruple region would correspond to the 'effective pairing segment'[13] or the 'switch area'[19]. Exchanges between chromosomes would be restricted to short segments; within the paired region, however, multiple exchanges could occur. This would lead to localized high negative interference. (4) The above mode of pairing by itself does not account for the observations on polarity. The possibility that polarized replication of DNA is responsible for the polarization of recombination has been suggested by several authors[18,20–22]. It is possible to imagine that recombination within the quadruple region is connected with DNA replication. However, this requires a detailed consideration of a model of exchange which is at this stage premature. We might just mention a possibility that arises in connection with the suggested mode of pairing. The paired molecules may separate by the unwinding of the quadruple helix. Alternatively the quadruple region may travel by the rotation of the two double helices. In either case pairing will be terminated in a sequential manner and could result in polarization of recombination.

Meselson and Weigle[23] have proposed a model for phage recombination which envisages pairing between fragments of parental chromosomes having a common segment of nucleotide sequence. The model successfully accounts for the genetic structure of the phage heterozygote and is also supported by some direct evidence[36]. The overlap region of this model could be in the form of the quadruple helix. When applied to other genetic systems Meselson's scheme has the disadvantage that it requires extensive fragmentation of the chromosomes prior to recombination. Pairing by localized denaturation could get over this difficulty.

The scheme of genetic pairing proposed above is not entirely novel. It is only an explicit statement of a possibility which has been suggested by others[23,31]. Although largely speculative, the model suggests, among other things, that when they first arise genetic exchanges must be closely associated with quadruple helices. Physical and quasi-genetic techniques which can test this predication are already available.

Summary

When F^- bacteria whose DNA is specifically labelled with ^3H-thymidine are mated to Hfr males, the genetic recombinants arising from such a mating contain labelled parental DNA. The association of 'old' DNA with 'new' genetic information argues in favour of a mechanism of recombination based on breakage and realignment of linear bonds between pre-existing DNA molecules. The amount of F^- DNA transferred to recombinants suggests that the breakage must involve both strands of the double helix.

The precision of crossing-over requires a site by site association between homologous segments of the chromosomes. This association or pairing is assumed to constitute a necessary condition for crossing-over. It is suggested that the specificity of pairing interaction, like the replicative and the coding specificity of DNA, resides in the hydrogen bonding properties of the four nucleotides. A model is considered according to which homologous segments of two-stranded DNA pair by the formation of localized quadruple helices. The exchange of similar strands between the two double-helices by a rearrangement renders this pairing specific. The localized quadruple helices provide the geometrical condition necessary for genetic exchange and serve to explain some additional features of crossing-over such as negative interference and polarity.

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Recent Studies on the Mechanism of Action of Polynucleotide Phosphorylase

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For several years the main effort of our laboratory has been directed toward investigating the properties of the enzyme polynucleotide phosphorylase. This enzyme was first discovered by Grunberg-Manago *et al.*[1] in extracts of *Azotobacter vinelandii.* The enzyme is widely distributed in bacteria and catalyses the reversible reaction shown in Equation 1 as well as various related reactions.

In n nucleoside-P-P $\overset{Mg^{2+}}{\rightleftharpoons}$ (nucleoside-P)$_n$ $+P_i$ . . . . (1) the forward direction (Equation 1) nucleoside diphosphate reacts to form an RNA[*]-like polymer of nucleoside monophosphate units plus inorganic phosphate. In the back direction polyribonucleotides are broken down in stepwise fashion by phosphorolytic cleavage of the internucleotide bonds. Several recent review articles summarize the extensive work carried out with this enzyme[2–5].

In general, each of the four common ribonucleoside diphosphates can serve separately as a substrate for the polymerization reaction, thus leading to the formation of the so-called homopolymers, that is, polymers containing only one base. However, experiments from many laboratories indicate atypical results when GDP is the substrate used. Thus, polymerization proceeds with great difficulty and partially purified enzyme preparations require suitable oligonucleotide primers in order to polymerize GDP even when such primers are not required for the polymerization of the other nucleoside diphosphates.

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[*] The following abbreviations are used: RNA, ribonucleic acid; P_i, inorganic phosphate; A, U, C, and G, adenylic, uridylic, cytidylic, and guanylic acid residues, respectively, in polyribonucleotides, thus, poly A is polyadenylic acid; NDP, nucleoside diphosphate.

TABLE 1—COMPOSITION OF POLYMERS MADE WITH POLYNUCLEOTIDE PHOSPHORYLASE

Polymer	Input ratio of nucleotides	Base ratio of polymer	Enzyme*	Ref.
AGUC	1 : 1 : 1 : 1	1 : 1·3 : 0·7 : 0·7	Az.	6
GU	1 : 1	3·2 : 1	Az.	7
GU	1 : 1	1·7 : 1	M	8
GU	0·33 : 1	0·63 : 1	M	8
GA	0·5 : 1	0·9 : 1	M	†
GC	1 : 1	10 : 1	M	†

*Az. indicates *Azotobacter agilis* enzyme and M indicates *Micrococcus lysodeikticus* enzyme.
†Jones, O. W. & Nirenberg, M. W., personal communication.

Mixtures of two or more nucleoside diphosphates lead to the formation of randomly arranged copolymers. As might be expected from the slow rate of polymerization of GDP alone, the rate of polymerization obtained when GDP is a co-substrate is less than the rate of reaction obtained with any other nucleoside diphosphate in the absence of GDP. We speak of this as an inhibition of the rate of polymerization by GDP although GDP is, of course, substrate. Indeed, it has been observed in several laboratories that in spite of the inhibition of rate of polymerization by GDP, GMP residues are actually incorporated into the copolymers preferentially. Thus, as indicated in Table 1, the relative amount of guanosine found in synthesized polymers is usually greater than the relative amount of GDP in the substrate mixture. The data in Table 1 summarize the results obtained in several laboratories and with enzyme prepared from two different bacterial sources.

Therefore, two matters require elucidation. First, the nature of the inhibition of polymerization rates by GDP and second, the reason for the enrichment of GMP units in polymers.

The experiments to be described were carried out with polynucleotide phosphorylase purified approximately 300-fold from extracts of *Micrococcus lysodeikticus*[9]. This preparation requires the addition of an oligonucleotide primer having an unesterified terminal C-3′ hydroxyl group for maximum rates of polymerization regardless of the nucleoside diphosphate used. Therefore, all experiments were carried out in the presence of approximately 0·5 mM triadenylic acid (pApApA) and polymerization was followed by measuring the release of P_i[10].

INHIBITION OF POLYMERIZATION BY GDP

The effect of GDP on the initial rate of ADP polymerization is shown in Fig. 1. The extent of inhibition of the overall rate of polymer formation

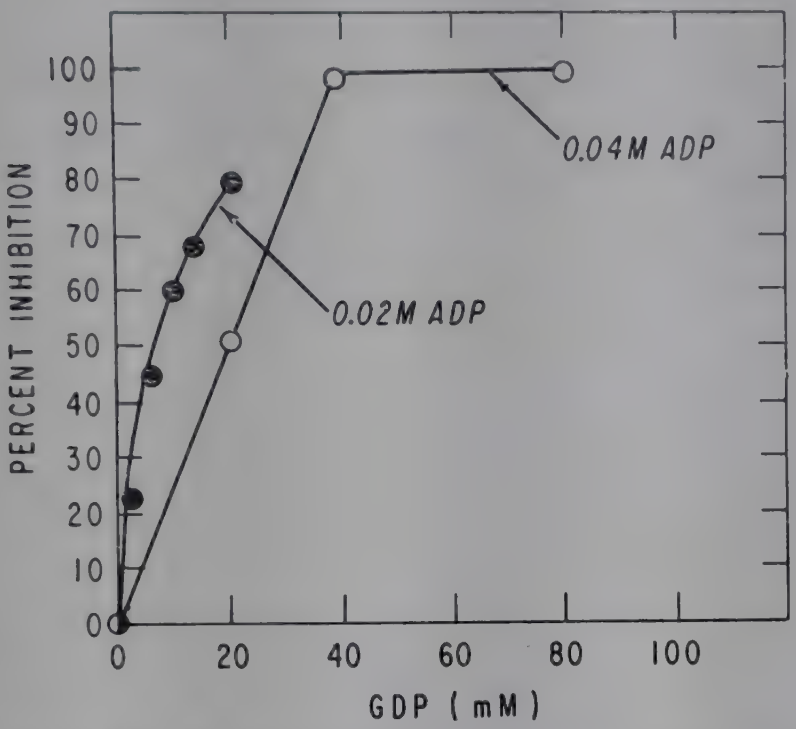

FIG. 1—EFFECT OF GDP ON ADP POLYMERIZATION: The percentage inhibition of the overall rate of polymer formation is plotted against the GDP concentration

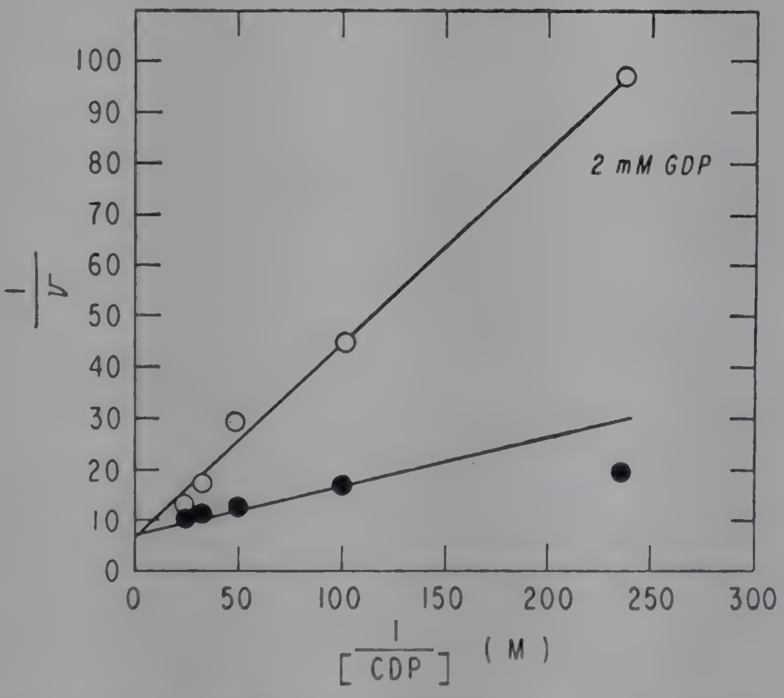

FIG. 2—POLYMERIZATION OF CDP IN THE PRESENCE OF GDP: Incubations were for 15 min. ● GDP; O, 2 mM GDP

depends both on the GDP concentration and on the ADP concentration, suggesting a competitive relation as might be expected. In the experiment shown in Fig. 2 the effect of GDP on CDP polymerization was studied. The data are plotted according to Lineweaver and Burk[11] and a competitive type inhibition is indicated. It should be pointed out again, however,

TABLE 2—EFFECT OF GDP ON RATE OF POLYMERIZATION

	Extent of homo-polymer formation	Effect of GDP on polymer formation* NDP : GDP		
	% NDP utilized	20 : 1	5 : 1	1 : 1
UDP	18	0	−25	−89
ADP	19	−26	−53	−81
CDP	17	−19	−88	−100

*Numbers represent the per cent decrease in the rate of polymerization caused by the presence of GDP. ADP, UDP and CDP were 10 m*M*. NDP represents nucleoside diphosphate.

that the GDP is not simply an inhibitor in these experiments. In the presence of two diphosphates the combined rate of reaction is being measured.

The data in Table 2 demonstrate again that the extent of inhibition by GDP depends on the GDP concentration. In this experiment ADP, UDP and CDP were 10 m*M*. The GDP concentration was varied to give ratios of ADP, UDP, or CDP, to GDP, of 20:1, 5:1 and 1:1. Inhibition is greatest at ratios of 1:1. The data also indicate that the extent of inhibition depends on the particular nucleoside diphosphate substrate. Thus, UDP polymerization is less sensitive to the presence of GDP than is ADP polymerization; CDP appears to be most sensitive.

The data in Figs. 3A and 3B compare the effect of GDP and ADP on the rate of CDP polymerization and also demonstrate again the effect of nucleoside diphosphate concentration on GDP inhibition. In this experiment the polymerization of CDP at 10 m*M* (Fig. 3A) and 40 m*M* (Fig. 3B) was studied. At 10 m*M* CDP, which is not saturating, GDP is strongly inhibitory; ADP stimulates the rate as one might expect. At 40 m*M* CDP, which is more than saturating, both GDP and ADP stimulate somewhat.

In order to determine the specificity of the inhibition by GDP, the effect of various purine and pyrimidine derivatives on ADP polymerization was investigated. Each compound was added at a final concentration of 5 m*M*; ADP was 10 m*M*. The following compounds gave no inhibition: inosine, guanosine, xanthosine, guanine, xanthine, adenine, 8-azaguanine, 2, 6-diaminopurine, 5′-AMP, 5′-GMP, CDP, UDP, and IDP. The four triphosphates ATP, UTP, CTP, and GTP are effective inhibitors as is 5′, 8-azaguanylic acid. ADP is self-inhibitory at concentrations above 0·02*M*.

Thus, the inhibition by GDP appears to be related to its role as a substrate. Inhibition has the characteristics of being competitive with other

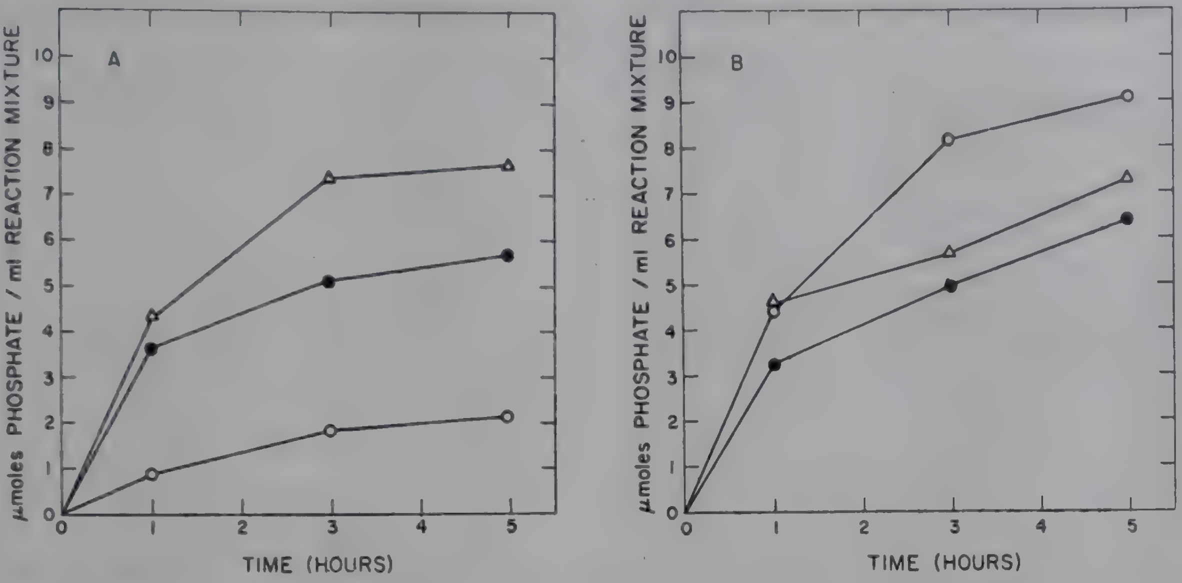

Fig. 3—Polymerization of CDP in the presence of ADP or GDP: ADP and GDP were 2 mM, (A) 10 mM CDP, (B) 40 mM CDP, ●, CDP; O, CDP plus GDP; △, CDP plus ADP.

substrates. It is dependent on the GDP concentration as well as the concentration of the other nucleoside diphosphate. Furthermore the extent of inhibition is related to the nature of the other diphosphate present in the reaction mixture. Inhibition is not caused by the guanine ring itself; in order to be effective the compound must be at least the nucleoside diphosphate.

Preferential incorporation of GMP units into polymers

Table 1 summarizes earlier results indicating a preferential incorporation of GMP units into polymer. Our experiments have confirmed these findings[10]. For example, a substrate mixture containing 10 mM CDP and 2 mM GDP (a ratio of CDP to GDP of.5 to 1), yielded a polymer with a C/G ratio of 2·6 to 1 (Table 3). We have found in addition, however, that conditions under which GDP is not strongly inhibitory may yield polymers with base ratios close to the original ratio of substrates. Thus, at 40 mM CDP and 2 mM GDP (CDP/GDP=20/1), no inhibition of CDP polymerization was noted (Fig. 3B, Table 3) and the C/G ratio of the polymer was 16 to 1 (Table 3). Thus, it appears that the preferential incorporation of GDP is most striking under the very conditions that afford GDP inhibition. This finding has been confirmed in our laboratory in many other experiments and also by Jones and Nirenberg in unpublished experiments.

Several possible explanations for the preferential incorporation of guanylic units into polymers were investigated.

If the affinity of polynucleotide phosphorylase was greater for GDP than for the other nucleoside diphosphates the preferential incorporation of GMP units could be explained. The available data do not, however, support this explanation. Because of the difficulties encountered in the polymerization

TABLE 3—INCORPORATION OF GUANYLIC ACIDS INTO POLY CG

CDP m*M*	GDP m*M*	Rate of polymerization*	Polymer base rate C : G†
40	0	3·3	—
40	2	4·5	16 : 1
10	0	3·6	—
10	2	0·8	2·6 : 1

*The rate of polymerization is expressed as the μmoles of inorganic P released per ml. of reaction in one hr.

†Base ratios determined by alkaline hydrolysis of isolated polymers followed by paper chromatographic separation of the components and quantitative elution[10].

of GDP itself it is difficult to obtain kinetic constants for GDP. K_m determinations have been made for another reaction catalysed by polynucleotide phosphorylase, namely, the arsenolysis of nucleoside diphosphates[12] (Equation 2).

$$\text{Nucleoside-P-P}^{32} \xrightarrow[\substack{\text{Mg}^{2+}\\ \text{pApApA}}]{\text{arsenate}} \text{nucleoside-P} + \text{P}_i^{32} \tag{2}$$

The values obtained for ADP, UDP, CDP and GDP were all similar and of the order of 10^{-3} *M* indicating no special preference for GDP[12].

We then investigated the relation between the base ratio of the isolated polymers and the extent to which the reaction had proceeded before polymer was isolated. In all previous works polymer had been isolated after polymerization had reached equilibrium. It seemed possible that the enrichment of guanylic residues might result from reactions proceeding at or near the equilibrium point. A reaction mixture containing 10 m*M* UDP and 2 m*M* GDP was incubated with enzyme. Poly UG was isolated from aliquots removed at various times during the course of the reaction. As shown in Table 4, the ratio of UMP units to GMP units in the polymer was the same regardless of whether 5% or 36% of the total nucleoside diphosphate had been converted to polymer. The latter point (36%) represented equilibrium for this reaction. The stoichiometry of this reaction was confirmed by chromatographic determination of the rate of disappearance of UDP and GDP. When AG polymers were prepared under similar conditions, however, the ratio of AMP to GMP in the polymer increased as the reaction proceeded toward completion and approached the input ratio of 5 to 1 (Table 4). Thus, in both cases, the tendency to incorporate GMP into copolymers preferentially is apparent even at the initial stages of the polymerization reaction.

TABLE 4—Composition of Total Polymer Product as Related to Extent of Polymerization

Polymer	Nucleoside diphosphate converted to polymer	Polymer base ratio U : G or A : G
	%	
UG	5	2·7
	12	2·4
	21	2·5
	35	3·1
	36	2·9
AG	15	2·8
	39	4·4

Reaction mixtures contained 10 mM UDP (or ADP), and 2 mM GDP. The polymer preparations were isolated by alcohol precipitation after deproteinization and were hydrolysed with alkali or acid. Hydrolysates were chromatographed on paper and the papers were eluted quantitatively to obtain base ratios[10].

The polymerization of nucleoside diphosphates by the *Micrococcus lysodeikticus* polynucleotide phosphorylase has a *p*H optimum of 9 or above. At *p*H 8·2 the reaction is slower. The relative preference for incorporation of GMP units into poly UG is, however, the same at *p*H 8·2 and at *p*H 9.

It has always been assumed that copolymers synthesized with polynucleotide phosphorylase contain a random arrangement of the different nucleotide residues. In several instances this has in fact been demonstrated[6,13]. However, in view of the results presented above, it seemed of interest to investigate the arrangement of the residues in the guanylic acid containing polymers. Therefore, a group of polymers were prepared using GDP labelled in the internal phosphate with ^{32}P and the polymers were subjected to nearest neighbour analysis[14]. The labelled GDP was synthesized according to the procedures of Tener[15] and Moffatt and Khorana[16]. The polymers were isolated and degraded with alkali to determine both the base ratios and the nearest neighbour frequencies[14]. The results are presented in Table 5 (poly UG) and Table 6 (poly AG). With the possible exceptions of polymers UG-I and UG-V, which contain relatively large amounts of guanylic residues, the polymers appear to be true random copolymers since the nearest neighbour frequencies agree well with those predicted for random arrangement of the nucleotide residues in the polymer. Thus, another possible explanation for the high level of incorporation of guanylic residues, namely, that a large number of essentially homopolymer chains were being synthesized, was eliminated. The addition of any particular

TABLE 5—NEAREST NEIGHBOUR FREQUENCIES OF POLY UG PREPARATIONS

Input (UDP/GDP)	5	5	20	20	1
Output (U/G)	2·0	2·7	6·6	7·8	0·5
GpG (found)	0·154	0·092	0·019	0·012	0·675
(random)	0·131	0·094	0·017	0·012	0·605
UpG (found)	0·208	0·215	0·114	0·099	0·104
(random)	0·231	0·212	0·115	0·098	0·173

TABLE 6—NEAREST NEIGHBOUR FREQUENCIES OF POLY AG PREPARATIONS

Input (A/G)	5·0	5·0
Output (A/G)	3·1	4·5
GpG (found)	0·062	0·037
(random)	0·063	0·037
ApG (found)	0·193	0·153
(random)	0·190	0·153

residue to the end of a growing chain appears to be a random event. The probability of that residue being GMP is dependent on the relative GDP content of the substrate mixture but is, in addition, increased by a still unknown factor.

We have adopted the working hypothesis that the various effects of GDP may be related to the well-known abilities of guanine mononucleotides[17] and polynucleotides[18,19] to form hydrogen-bonded complexes. Recent experiments have been designed to investigate this hypothesis and preliminary results are presented below.

Effect of elevated temperature and urea on polynucleotide phosphorylase

Our approach has consisted of investigating the effects of heat and of urea on the rate of enzymically catalysed reactions and in particular of reactions involving GDP. The first group of experiments are concerned with the polymerization reaction and again the reaction is carried out in the presence of triadenylic acid as primer. The extent of reaction is followed by measuring the release of inorganic phosphate.

As shown in Table 7, the *M. lysodeikticus* polynucleotide phosphorylase is relatively unstable at elevated temperatures. When the polymerization of UDP is followed at 37°, 45° and 50°C., the instability is noticeable as a decrease in the extent of reaction at 30 min. with increasing temperature.

If a relatively low concentration of GDP is included in the reaction mixture, the expected inhibition of the reaction at 37°C. is readily seen (Table 7). However, as the temperature is elevated this inhibition disappears and at 50°C., there is actually a stimulation of the rate of polymerization over that obtained in the absence of GDP. The stimulation appears to result from a protection of the enzyme from the effects of heat in the presence of GDP. GMP will not replace GDP in this function.

The graph in Fig. 4 shows a similar but more extensive experiment. Both in the presence and absence of GDP the reaction rate increases with increasing temperature from 26° to 37°C. Above 37°C. the sensitivity of the enzyme to heat is apparent as is the protection by GDP.

TABLE 7—Effect of Temperature on Polymerization

Nucleoside diphosphate	μmoles P_i released/ml. reaction		
	37°C.	45°C.	50°C.
UDP	3·0	2·2	0·7
UDP+GDP	1·8	2·2	1·6

UDP, 10 m*M*; GDP, 4 m*M*.
Incubation time, 30 min.

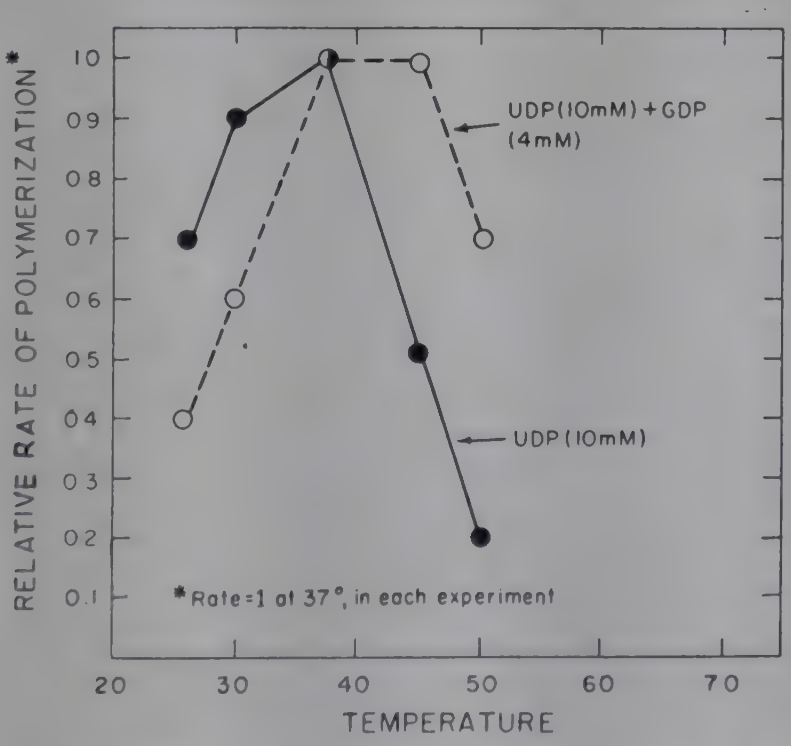

Fig. 4—Effect of temperature on polymerization: Incubations were carried out for 30 min. In each experiment the μmoles of P_i released at 37°C. was set equal to 1, although they were not equal (*see* Table 7)

Table 8 compares the sensitivity of the enzyme to temperature in the presence of UDP, ADP, or CDP. At 10 m*M* nucleoside diphosphate, UDP affords the least protection, CDP the most. Increasing the nucleoside diphosphate concentration to 20 m*M*, which should ensure saturation of the enzyme, markedly increases the stability of the enzyme with each of the diphosphates. It is of interest to point out that at 20 m*M* UDP, 4 m*M* GDP no longer increases the stability of the enzyme and doubling the GDP concentration has no effect on this.

It is clear that the type of experiments described above are difficult to interpret because of the steady loss of activity at elevated temperatures during the course of the experiments. We, therefore, attempted to carry out preincubation studies. Enzyme was incubated at elevated temperatures with various additions and then the reaction was started by the addition of the remaining components. No evidence for protection of enzyme by any of the components could be obtained. If anything the data suggest that only when all the components are present, and the reaction is actually proceeding, is GDP able to protect the enzyme from the effect of elevated temperature.

A second approach to the question of the relation between secondary structure and the effect of GDP on polynucleotide phosphorylase has involved the use of urea. The results are similar to those obtained at elevated temperatures. Thus, the rate of UDP polymerization is quite sensitive to increasing

TABLE 8—EFFECT OF VARIOUS NUCLEOSIDE DIPHOSPHATES ON TEMPERATURE SENSITIVITY OF POLYNUCLEOTIDE PHOSPHORYLASE

Nucleoside diphosphate concentration	Nucleoside diphosphate	37°C.	50°C.	50°C./37°C.
m*M*		μmoles P_i released/ml. reaction		
10	ADP	2·4	0·8	0·33
	CDP	2·7	1·5	0·56
	UDP	2·6	0·6	0·23
	UDP+GDP (4)*	1·2	0·7	0·58
20	ADP	2·8	1·9	0.68
	CDP	3·0	2·7	0·90
	UDP	2·8	1·2	0·43
	UDP+GDP (4)*	1·6	0·5	0·31
	UDP+GDP (8)*	0·6	0·2	0·33

*Numbers in parenthesis are m*M* concentrations of GDP.

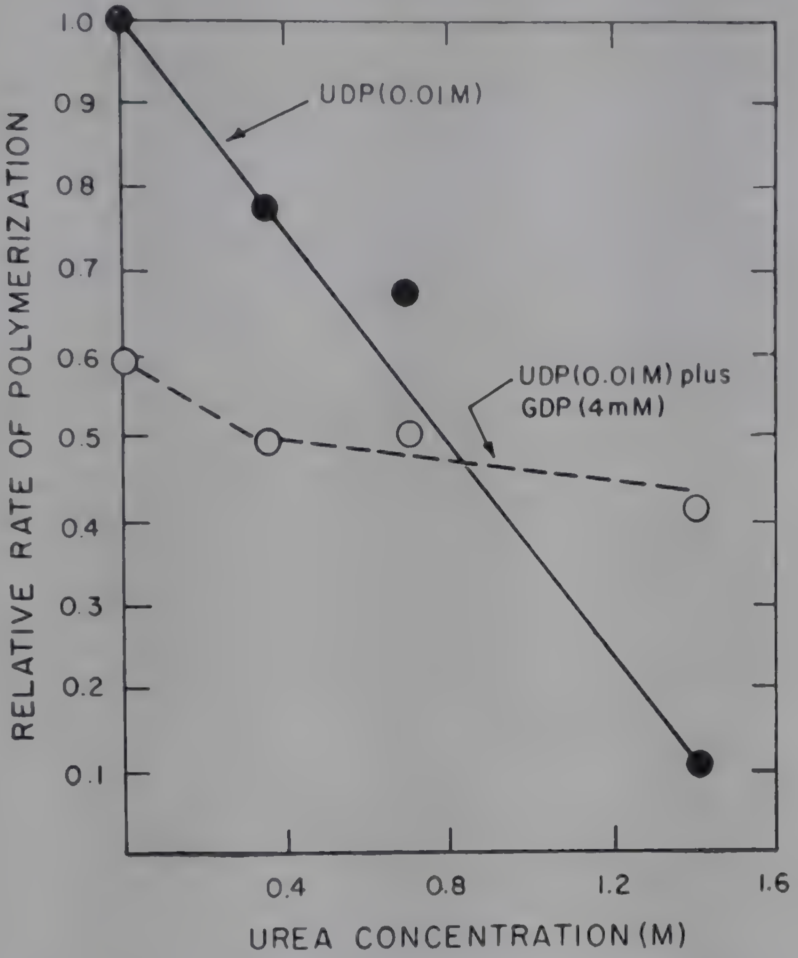

FIG. 5—EFFECT OF UREA ON UDP AND UDP+GDP POLYMERIZATION: Incubation time was 30 min. The extent of polymerization of UDP alone, in the absence of urea, is set equal to 1

concentrations of urea and is inhibited 90% at 1·4 *M* urea (Fig. 5). The presence of GDP appears to protect the enzyme from inactivation by urea (Fig. 5). The polymerization of 10 m*M* UDP plus 4 m*M* GDP is almost insensitive to the presence of 1·4 *M* urea.

This effect of urea is also seen with other nucleoside diphosphates. An experiment with ADP is shown in Table 9. At 37°C. ADP polymerization is inhibited by 0·7 *M* urea, but when ADP and GDP are present urea is not inhibitory. When the reaction is run at 45°C. in the absence of urea the usual protection by GDP is apparent. Furthermore, when the reaction is carried out at 45°C. in the presence of 0·7 *M* urea ADP polymerization is inhibited almost 90%, while the ADP plus GDP mixture reacts at a good rate. Thus, the effects of elevated temperature and urea appear to complement one another and GDP is able to protect each one.

Although we anticipate that these results are related to the odd characteristics of GDP as a substrate for polynucleotide phosphorylase, certain questions must be investigated before any reasonable interpretation of these data can be made. For example, what is the base ratio of copolymers containing guanylic acid when reactions are run at elevated temperatures?

TABLE 9—Effect of Temperature and Urea on Polymerization

Nucleoside diphosphate	Temp. °C.	µmoles P_i released/ml. reaction	
		−Urea	+Urea (0·7 *M*)
ADP	37	2·6	2·0
ADP	45	2·7	0·3
ADP+GDP	37	1·4	1·7
ADP+GDP	45	2·3	1·4

ADP was 10 m*M*; and GDP 4 m*M*.

What is the effect of urea and of elevated temperature on the physical characteristics of the enzyme under various conditions ? Current work in our laboratory is directed towards obtaining data on these points.

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Nucleolar Ribonucleic Acid

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This paper deals with two experimental approaches to the function of nucleolar RNA. First, an operational approach to find the interrelation between nucleolar and chromosomal RNA, that is, the genetic principle of nucleolar function. Second, a biophysical approach to characterize the molecular species in nucleolar RNA.

BIOLOGICAL MATERIAL AND TECHNIQUE

The cells studied are of salivary glands in larvae of the midge *Smittia parthenogenetica*. The cells number about fifty in each gland of a pair, and are not obviously regionally specialized as in other midges. The nucleus contains three giant polytene chromosomes, one of which has a well-recognizable nucleolus organizer surrounded on all sides by the nucleolus: the morphological arrangement of these large structures (the diameter of the nucleolus surpasses 30μ in squash preparations) are favourable for the localization of syntheses with tritium autoradiography with a resolution of 1μ.

The glands used in all experiments (except in section A) are taken from larval stage III (ref. 1) just before metamorphosis, when they have become very voluminous and secrete glue for the puparium. As revealed by electron microscopy, the cells are fully differentiated (Jacob, J., unpublished observations). The glands remain functional for several hours of *in vitro* incubation in a synthetic medium[2]. This is shown by the incorporation of precursors into cell RNAs[1] and by the release of ^{35}S-labelled secretion[3]. Thus, cells can be studied *in vitro* within a functional organ.

Experimental details are fully described elsewhere[2]. Autoradiography was exclusively used in sections A-D. The procedure previous to autoradiography adheres as much as possible to conventional biochemical procedures. In the experiments using autoradiography treatments were carried out on glands incubated individually at 25°C. The treatments were titrated to yield visual differences in the autoradiographs generally greater than 30% with respect to controls. Label is estimated in treated cells after an adequate exposure which is determined in controls. The autoradiographs illustrated in the figures for each pair of treated and control glands were identically exposed. All tritiated ribonucleosides are incorporated almost entirely into ribonuclease-sensitive materials in the nucleus, and incorporation in cytoplasm just begins.

Experiments and Results

(A) **Experiments with thymidine**

Incorporation of thymidine is a function of the amount of DNA undergoing replication, and can reveal smaller amounts of DNA than conventional cytochemical techniques. Stage III, at the end of the 4th (last) larval instar, is the last stage in which chromosomes including the nucleolus organizer incorporate large amounts of thymidine into DNA *in vitro*, though nucleoli fail to incorporate[4]. DNA replication was now searched for in younger larval stages in which early nucleolar growth is occurring.

Glands of four stages were studied: (1) the earliest stage (1.5 mm.) after hatching (0·5 mm.) from which glands could be dissected free-hand; (2) an intermediate stage (2·2 mm.) between the first and third stages; (3 & 4) stages I and II (ref. 1) corresponding to 3rd and early 4th instars, respectively. To give an idea of the growth of the nucleus, its diameter increases from 6μ in stage I to 50μ in stage IV. Glands were incubated for 6 hr with tritiated thymidine (Schwarz Bio Research; 360 mC/mM, 40 μC/ml.). The autoradiographs were exposed until blackening of the emulsion reached saturation above chromosomes. In all four stages nucleoli as weakly labelled as cytoplasm were seen in nuclei giving a strong response (Fig. 1) (the non-chromosomal label was most probably due to some conversion of thymidine to uridine). Strongly labelled nucleoli in otherwise less labelled nuclei were not found. Assuming that any existing nucleolar DNA replicates *pari passu* with chromosomal DNA, the observations tend to show that the amount of nucleolar DNA can possibly be only minute.

(B) **Action of actinomycin and proflavin on nucleolar RNA**

We started using actinomycin C in early 1961 (ref. 5, p. 323, and ref. 6), following reports that the antibiotic attacked nucleoli[7]. Later it was found that actinomycin interfered directly with primer DNA for RNA polymerase[8]. Pretreatment for 60 min. with actinomycin D (0·5-1·0μg./ml., $3{\cdot}9\text{-}7{\cdot}8 \times 10^{-7} M$) suppresses incorporation of tritiated uridine into nucleolar RNA[4]. These experiments have now been confirmed with tritiated guanosine and cytidine. The low dose of actinomycin (i.e. concentration and time) is in line with a direct effect on primer DNA. Primer for DNA polymerase was unaffected as expected, as shown by the normal chromosomal uptake of thymidine[4]. The direct effect of actinomycin is supported

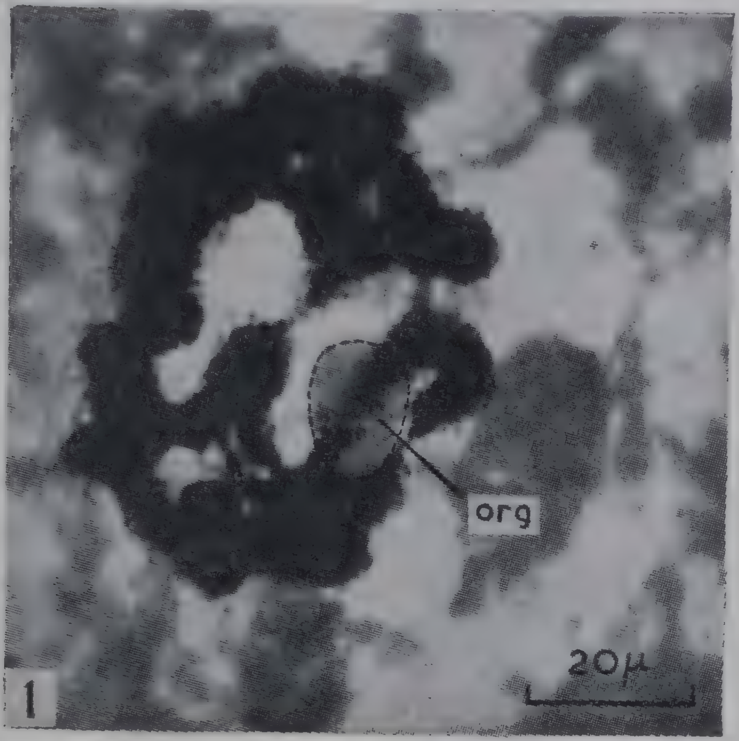

FIG. 1—AUTORADIOGRAPH OF SQUASHED SALIVARY GLAND (STAGE II) AFTER INCUBATION WITH ^{3}H-THYMIDINE: Only chromosomes including the nucleolus organizer (org) are highly labelled. Nucleolus outlined

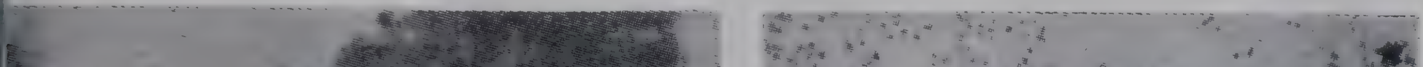

FIG. 2—AUTORADIOGRAPHS OF CONTROL (A) AND ACTINOMYCIN-TREATED GLANDS (B): Glands were incubated first with ^{3}H-guanosine (0·125 m*M*) and adenosine (0·1 m*M*), rinsed and then incubated for 1 hr in standard medium alone (controls) or with 1 µg./ml. actinomycin D. Actinomycin reduced RNA label more in chromosomes than in the nucleolus (no). Residual incorporation during the second incubation is shown by the labelled cytoplasm

by the identical effect of pretreatment for 20 min. with proflavin ($1{\cdot}4 \times 10^{-5}$ M; ref. 4), which we used at the suggestion of J. Hurwitz. The acridine affects primer for RNA polymerase at a higher dose than actinomycin[8].

Of many substances tested so far only actinomycin and proflavin cancel RNA synthesis in nucleoli earlier than in chromosomes. For instance, thioacetamide affects nucleolar synthesis less preferentially[6]. Mitomycin C, which attacks DNA, inhibits nuclear synthesis in general (Jacob, J. & Sirlin, J. L., unpublished data). Conversely, other substances in section D affect chromosomal synthesis earlier than nucleolar synthesis.

Actinomycin was reported to depolymerize *in vivo* high molecular weight RNA directly to low molecular weight components while leaving soluble RNA practically intact[9]. This was tested by incubating for 1 hr with actinomycin glands that had been previously incubated with tritiated guanosine and non-radioactive adenosine. The internal RNA label was generally more reduced by the antibiotic in chromosomes than in the nucleolus (Fig. 2). The greater reduction in chromosomes is more probably attributable to depolymerization of previously labelled RNA than to inhibition by actinomycin of the residual incorporation during the second incubation, since in this case the nucleolus would have been more inhibited than the chromosomes[4]. By the same token, loss of labelled RNA by turnover during the second incubation probably would also have been greater in the nucleolus than in the chromosomes, since actinomycin inhibits synthesis in the nucleolus earlier and more completely than in the chromosomes[4]. The observations in fact correspond with what could be anticipated from a predominant new synthesis of sRNA in the nucleolus (section E).

(C) Effect of nucleosides on the action of actinomycin

Actinomycin depresses *de novo* synthesis of purines[10], possibly as a secondary effect of the inhibition of RNA synthesis. The antibiotic does not interfere with formation of ribonucleotides from preformed purines; in fact, these ribonucleotides increase[10,11]. However, these observations were made on whole cells and might not reveal a depletion restricted to a nucleolar pool. This is true even after it was shown that actinomycin labelled with tritium or carbon (Harbers, E., personal communication) is not bound to the nucleolus after loss of water-soluble compounds was prevented. Experiments were, therefore, aimed at supplementing the precursor pools by addition of nucleosides during and after treatment with actinomycin.

Experiment 1. Glands were incubated for 1 hr with actinomycin D (0·16, 0·5 and 1·3μg./ml.), rinsed twice during 10 min., and then incubated for 25 min. with tritiated guanosine and non-radioactive adenosine, cytidine and uridine (all four 0·1 mM). Controls were similarly incubated without antibiotic. Synthesis of nucleolar RNA was progressively inhibited from 0·16 to 0·5μg. (Fig. 3), and at 1·3 μg. the inhibition was severe.

Experiment 2. Glands were incubated for 1 hr with actinomycin

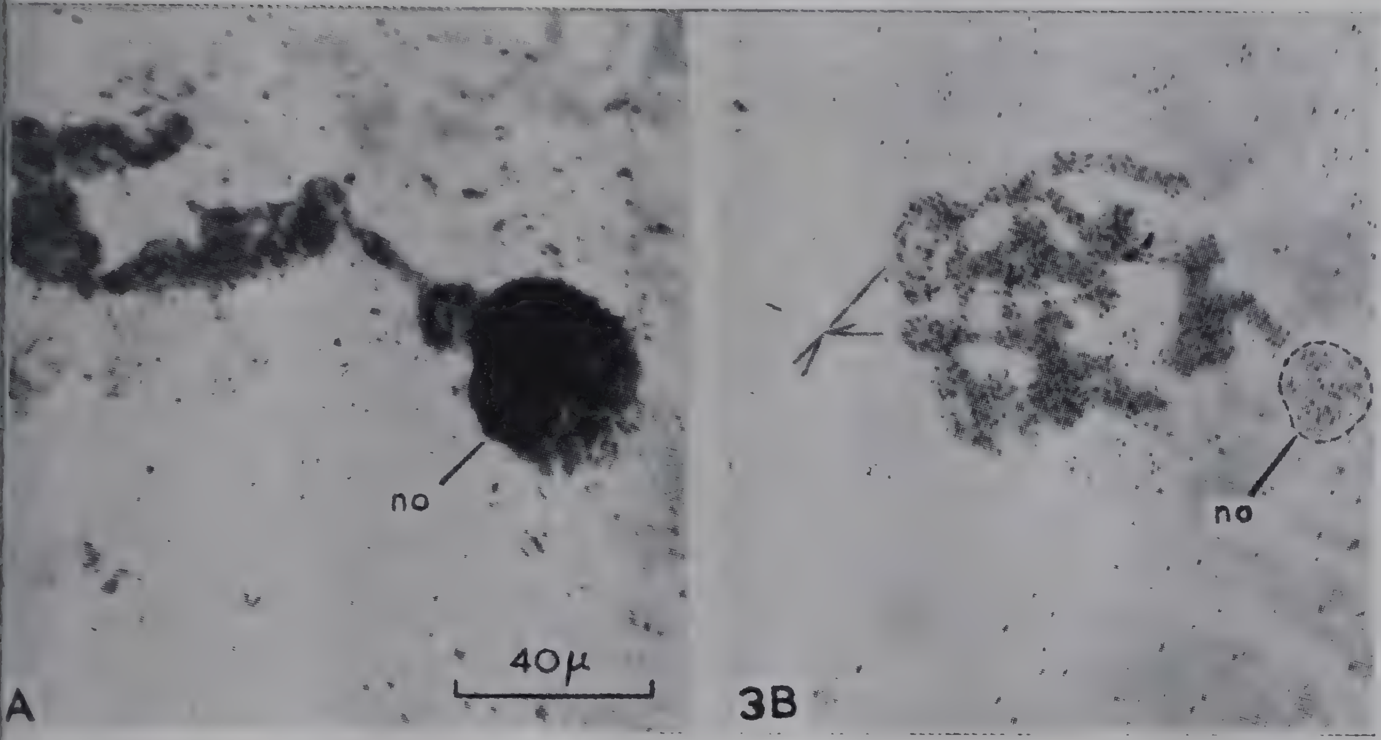

Fig. 3—Autoradiographs of control (A) and glands treated with 0·5 μg./ml. actinomycin (B) before and during incubation with ^{3}H-guanosine in the presence of unlabelled nucleosides (*see* text): The nucleosides do not counteract the actinomycin inhibition of RNA synthesis, which is more severe in the nucleolus than in chromosomes. Nucleolus (no) outlined in B

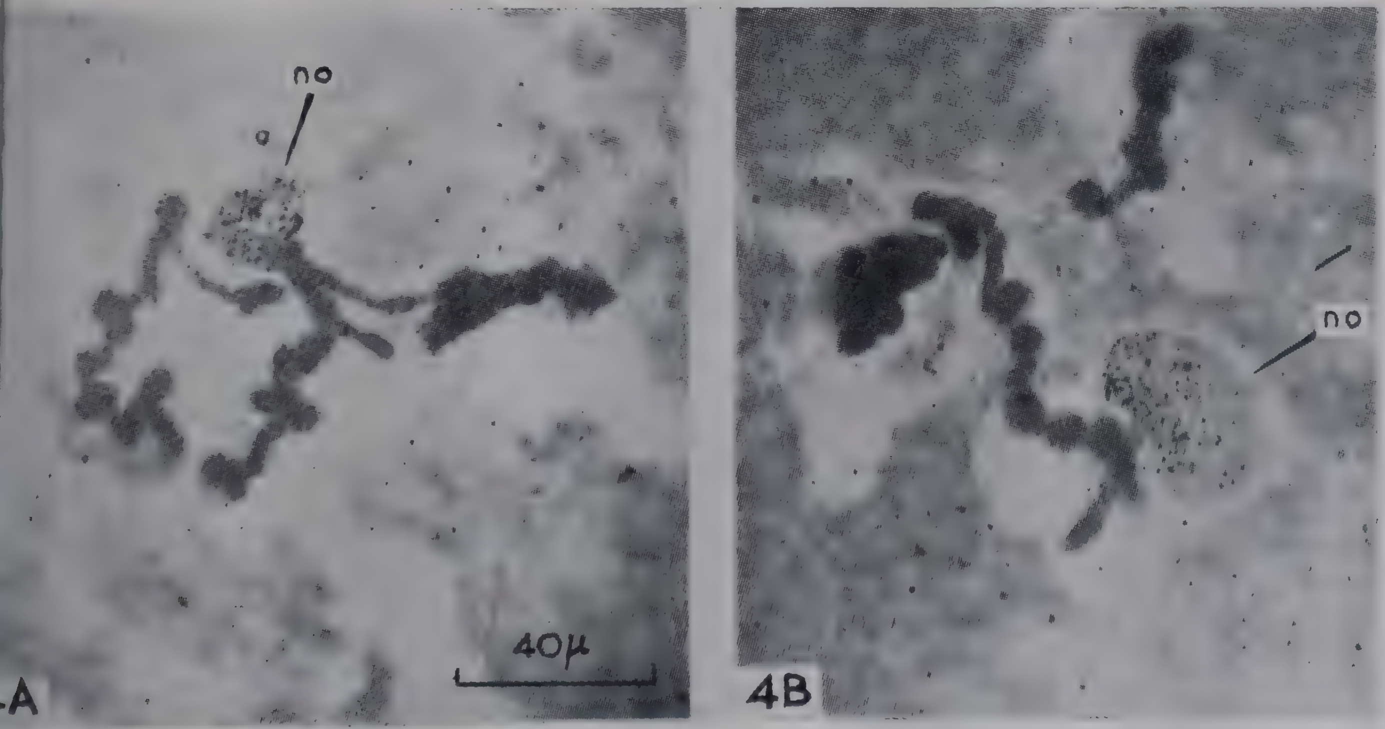

Fig. 4—Autoradiographs of control (A) and glands treated with 1·3 mg./ml., benzamide (B) before and during incubation with ^{3}H-uridine: RNA synthesis is more inhibited in chromosomes than in the nucleolus (no). Chromosomes are often damaged with this dose of benzamide

(1·0 μg./ml.), rinsed for 10 min., and incubated for 30 min. with tritiated uridine (0·05 m*M*) and non-radioactive adenosine, cytidine, guanosine, deoxyguanosine and thymidine (all 0·1 m*M*). Controls were similarly incubated but without antibiotic. Nucleolar RNA synthesis was totally inhibited by actinomycin.

Experiment 3. Glands were incubated for 1 hr with actinomycin (2·0 μg./ml.) and non-radioactive adenosine, cytidine, guanosine, deoxyguanosine and uridine (all 0·05 m*M*). The glands were then rinsed and incubated for 25 min. with tritiated guanosine (0·1 m*M*) and non-radioactive deoxyguanosine (0·025 m*M*), adenosine, cytidine and uridine (all 0·05 m*M*). Controls were similarly incubated without actinomycin. Nucleolar synthesis was totally inhibited.

The concentration of actinomycin was chosen to allow the simultaneous presentation of nucleosides at high concentration and at a molar ratio of each nucleoside to antibiotic of approximately 30 : 1. At this ratio the binding of these molecules reaches an equilibrium[12] at which most nucleoside and a proportion of actinomycin remain unbound. For actinomycin this is clear from the nucleolar inhibition.

Experiment 4. Glands were incubated for 1 hr with actinomycin (0·5 μg./ml.) and non-radioactive deoxyguanosine (0·076 *mM*) or guanosine (0·076 and 0·133 *mM*), and then for 30 min. directly in the same media as before with tritiated uridine. Controls were similarly incubated with only actinomycin, or guanosine (0·133 *mM*), or none. Deoxyguanosine counteracted the actinomycin inhibition almost entirely. Guanosine did partly counteract the inhibition at the high concentration but not at all at the low concentration.

The aim was to compare the competition with actinomycin by deoxyguanosine at a molar ratio 1 : 200, and by guanosine at 1 : 200 and 1 : 350. From previous experiments on cells[12] it was expected that deoxyguanosine would combine with most antibiotic, while guanosine would approach this condition at the higher ratio only. The observations agree well with expectation.

CONCLUSION

All nucleosides are known to enter the cells when offered as tracers at generally low concentration. Depletion of cell precursor pools would prevent RNA synthesis, and supplementation of the medium with a high concentration of nucleosides should restore it, except if pools are tightly compartmented. If such a restoration of synthesis is not observed, since the antibiotic does not bind nucleosides appreciably[12], it must inhibit RNA synthesis through some mechanism other than interference with internal pools. In experiments 1 & 2 the inhibition persists after withdrawal of actinomycin, even when the nucleosides are present before withdrawal (Experiment 3). This evidence suggests that actinomycin does not inhibit nucleolar synthesis by depleting pools.

Experiment 4 shows that deoxyguanosine alleviates the actinomycin inhibition effectively at molar ratios of 200: 1. Guanosine, the physiological RNA precursor, is less effective even at ratios of 350: 1, as had been anticipated from its lesser binding affinity to antibiotic *in vitro*[12]. Although the binding with actinomycin occurs outside cells, more unbound guanosine than deoxyguanosine would have been available to the cells throughout incubation.

(D) Preferential inhibition of chromosomal RNA synthesis

Screening of substances that would act in the opposite direction to actinomycin and proflavin, namely, inhibit chromosomal synthesis earlier than nucleolar synthesis, was started about two years ago in our laboratory. Observations on benzamide and DRB, which have this effect, are described in some detail as they may interest other workers. Characteristics of the individual pools and/or synthetic systems involved in these inhibitions are under study.

Benzamide. The preferential inhibition of chromosomal synthesis was discovered when studying the anti-hormone action of benzamide in endocrines of insect larvae[13]. The mechanism is not known.

Glands were incubated for 2 hr with benzamide (0·25, 0·75 and 1·3 mg./ml.) and then incubated for 30 min. in the same medium with tritiated uridine. Controls were similarly incubated without benzamide. Chromosomal RNA synthesis was inhibited partially at 0·75 mg. and completely at 1·3 mg., while some inhibition of nucleolar synthesis was apparent only at 1·3 mg. (Fig. 4).

In a time-sequence experiment, glands were incubated with benzamide (1·3 mg./ml.) for 0·5, 1, 2, 3 and 4 hr, and then for 30 min. in the same medium with tritiated uridine. Controls were incubated for 0·5 and 4 hr without benzamide. In this experiment inhibition of chromosomal RNA synthesis was practically complete from 0·5 hr onwards. Inhibition of nucleolar synthesis was about 50% at 0·5 hr and increased slowly afterwards. This experiment reveals a different inhibition kinetics in chromosomes and nucleoli.

DRB. This purine riboside analogue, 5-6-dichloro-l-(β-D-ribofuranosyl) benzimidazole, and the trichloro-substituted derivative (TRB), were reported to inhibit RNA synthesis in general[14]. We previously reported preferential chromosomal inhibition for TRB[6].

Glands were incubated for 2 hr with DRB (10, 40, 80 and 150 μg./ml.; the latter did not dissolve completely at 37-40°C.), and then for 30 min. in the same medium with tritiated uridine. Controls were similarly incubated without DRB. At 10 μg. some chromosomal RNA was synthesized but none at higher concentrations. Nucleolar synthesis was partly impaired only at 80 μg. but severely at 150 μg.

The previous experiment was repeated using DRB (20μg./ml.) and tritiated uridine (0·013 mM), with non-radioactive cytidine (0·1 mM) present throughout. In a parallel experiment tritiated guanosine (0·125 mM) was used with non-radioactive adenosine (0·1 mM) present throughout. Controls for both experiments had no DRB. Chromosomal uptake of uridine was suppressed (Fig. 5) and of guanosine was strongly inhibited (the uptake in guanosine controls was very high). Synthesis of the internally labelled RNA was much less affected in nucleoli than in chromosomes.

In a time-sequence experiment, glands were incubated with DRB (at saturation, <200 μg./ml. at 20°C.) for 0·5, 1·5 and 4 hr, and then for 30 min. in the same medium with tritiated uridine. Controls were incubated for the same periods without DRB. Chromosomal synthesis stopped already after the shortest treatment. Nucleolar synthesis was reduced to 70% after 0·5 hr and was less than 50% after 4 hr. As in the comparable experiment with benzamide, a different kinetics is shown by chromosomes and nucleoli.

Benzamide, DRB and TRB combined. The three substances were used together to inhibit nucleolar RNA synthesis which resisted the action of benzamide and DRB separately.

Glands were incubated from 15 min. up to 6 hr with benzamide (1·5 mg./ml.), DRB and TRB (saturated mixture made with 150 μg./ml. each at 37-40°C.), and then for 30 min. with tritiated uridine in the same medium. Controls had similar incubations without inhibitors. Synthesis in chromosomes stopped as from 15 min., while in the nucleolus it was somewhat inhibited at 15 min. and stopped at 30 min.

The previous experiments were repeated incubating first for 30 min. with benzamide and DRB, or benzamide and TRB, or DRB and TRB, at the same concentrations as before. It was found that to stop nucleolar synthesis the presence of benzamide in the mixture is essential, and that TRB is probably more important than DRB.

Glands were incubated for 50 min. with the previous triple mixture, and then for 30 min. with tritiated uridine alone. Controls were similarly incubated without inhibitors. Both nucleoli and chromosomes incorporated more than controls, indicating that RNA synthesis is rapidly restored after withdrawal of inhibitors.

(E) Characterization of newly synthesized nucleolar RNA

In the preliminary experiments reported here the RNA synthesized in the presence of an inhibitor of chromosomal synthesis (DRB) was compared with RNA synthesized in its absence. It was found that the nucleolar RNA synthesized in the presence of DRB was sRNA.

Glands were collected from several mass incubations and quick-frozen for storage. RNA was extracted[11] and precipitated in the presence of enough

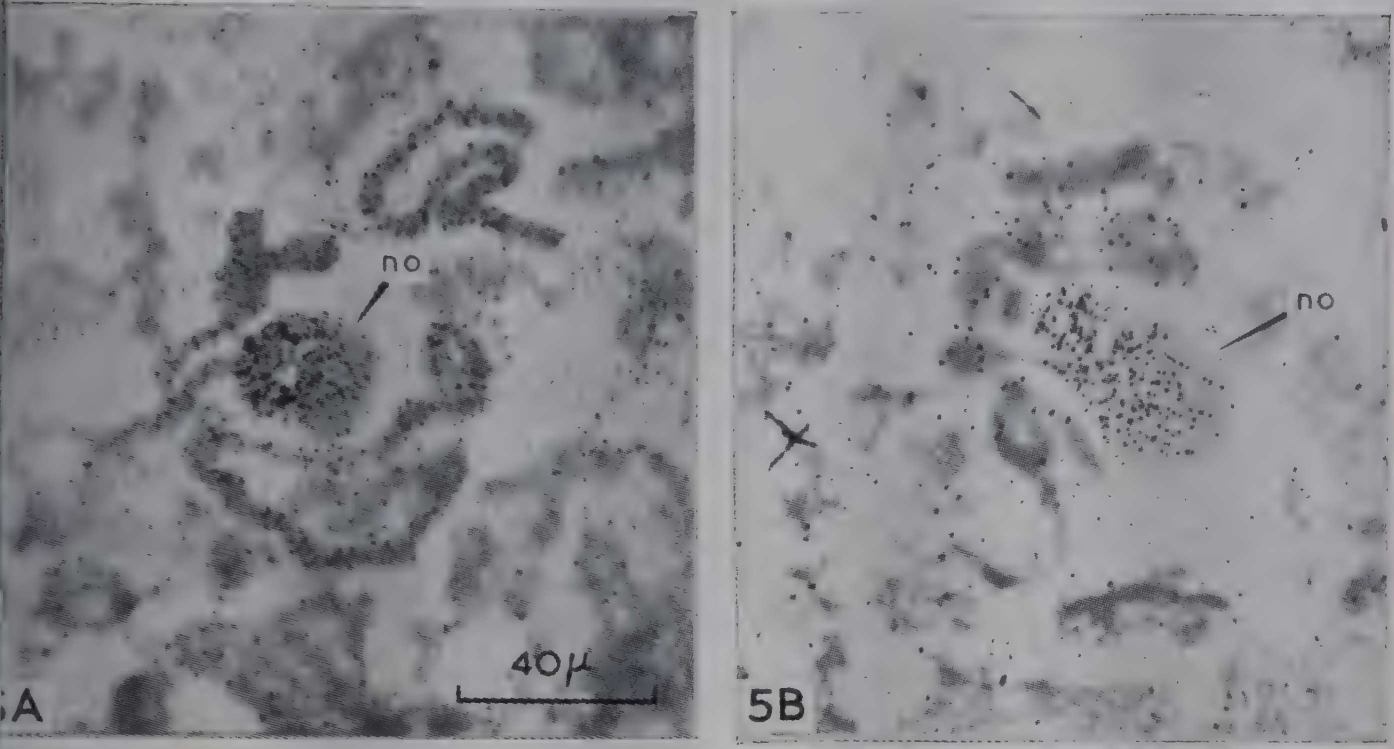

Fig. 5—Autoradiographs of control (A) and glands treated with 20μg./ml. DRB (B) before and during incubation with ^{3}H-uridine (0·013 m*M*): Unlabelled cytidine (0·1 m*M*) was present throughout. Chromosomal RNA synthesis is more inhibited than nucleolar synthesis

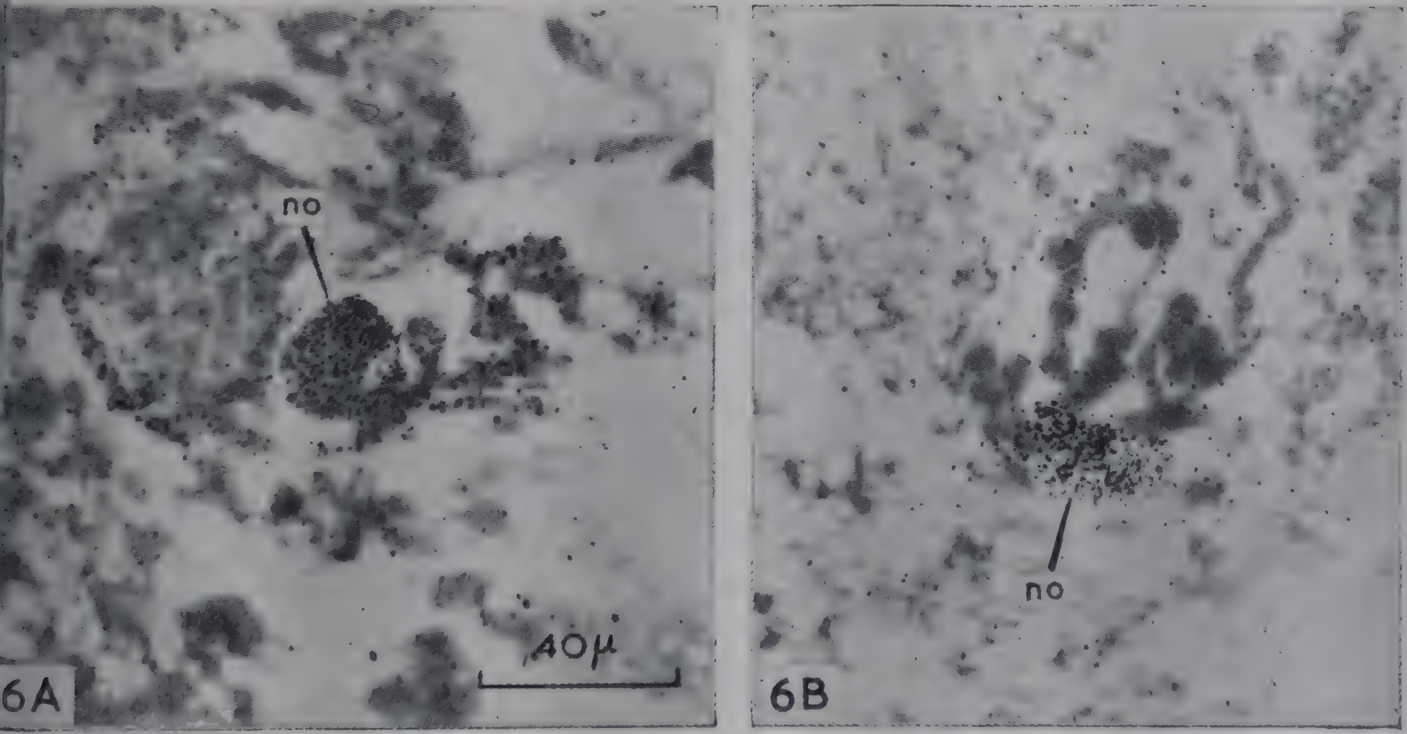

Fig. 6—(A) Autoradiograph of gland incubated with ^{3}H-guanosine only: The chromosomes and the nucleolus (no) are labelled. (B) Autoradiograph of gland treated with 40 μg./ml. DRB before and during incubation with ^{3}H-guanosine: Most of the label is in the nucleolus. In both figures cytoplasm shows background only

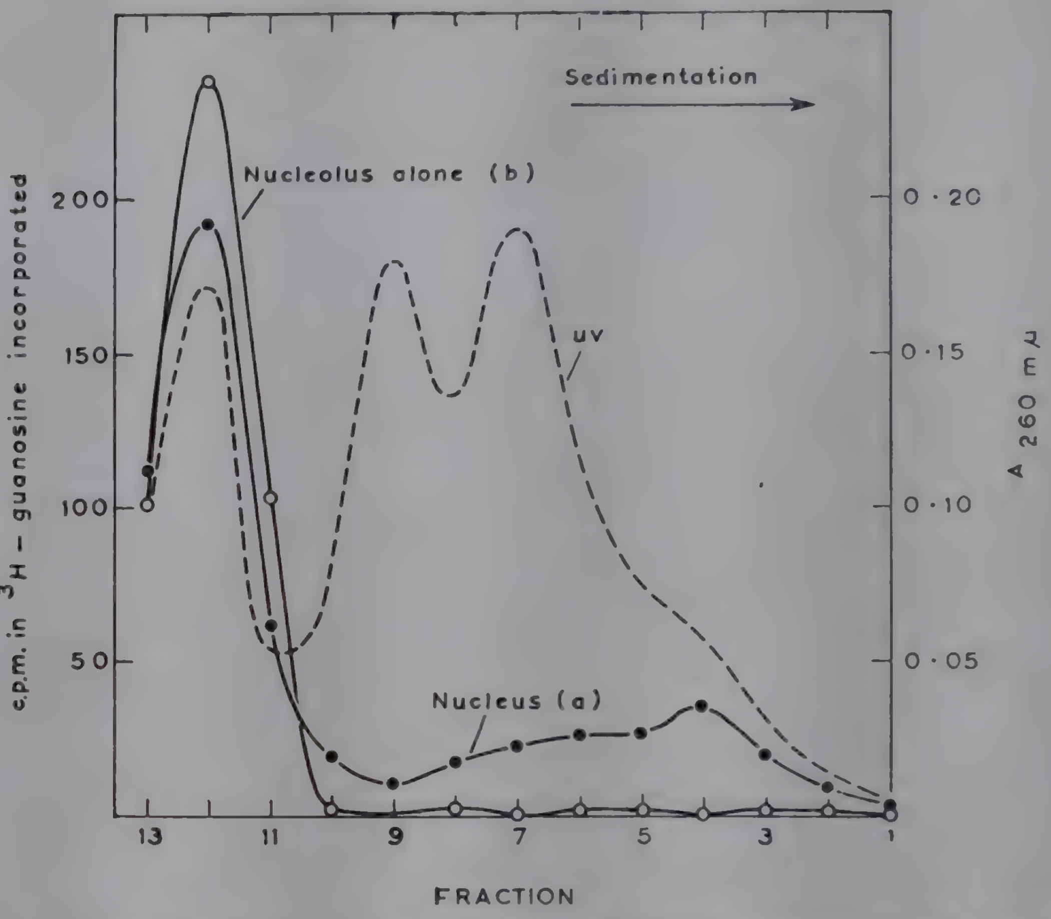

FIG. 7—BAND CENTRIFUGATION OF ^{3}H-GUANOSINE LABELLED SALIVARY GLAND RNA: The glands were incubated and the RNA extracted as described in the text. The ultraviolet pattern shows the sRNA (4-5 S) and the two ribosomal peaks (18 and 28 S). A shoulder at 40 S is reproducibly obtained. Radioactive samples with 20 or more c.p.m. above background were counted to an accuracy better than 10%. Curve (b) shows that nucleolar RNA extracted from glands which had incorporated tracer in the presence of DRB yields counts in the sRNA region only. Curve (a) shows that RNA of control glands contains an important proportion of counts in the ~ 40S region as well. Autoradiographs of glands from these incubations are shown in Fig. 6

carrier pea RNA to ensure recovery. In the experiment with DRB 70-80% of the incorporated radioactivity was recovered in the RNA fraction; the radioactivity in the residue was also in RNA as found by hydrolysis. The RNA was fractionated by band centrifugation through 2*M* CsCl[16] in a Spinco SW 39 (35,000 r.p.m.; 85 min.). The distribution of radioactivity was measured in a Packard scintillation counter.

RNA synthesized in the presence of DRB. Two hundred and sixty pairs of glands were incubated at 25°C. first for 2 hr with DRB (40 μg./ml.) and then for 45 min. in the same medium with tritiated guanosine (50 μC/ml.). Autoradiographs made of these glands showed that most of the radioactivity originated in nucleoli (Fig. 6B). Separation of the extracted RNA into its molecular species revealed that the sRNA peak contains virtually all the radioactivity, while the radioactivity in high molecular weight peaks is not significant (Fig. 7B).

RNA synthesized in the absence of DRB. Four hundred and thirty pairs of glands were directly incubated for 1 hr with tritiated guanosine (100 μC/ml.). Autoradiographs showed the normal distribution of label in the nucleolus and chromosomes (Fig. 6A). Separation of the RNA showed that, apart from the radioactivity in the sRNA peak, a considerable portion was recovered in the 40 S region (Fig. 7A).

Discussion

A probable interpretation of the observations with actinomycin (section B) is that nucleolar RNA synthesis is primed by DNA. The dose of actinomycin and the expected higher dose of proflavin which suppress nucleolar synthesis are in line with a specific action on primer DNA. However, the secondary effect of actinomycin in depolymerizing RNA suggested by our observations (section B) cannot exclude the alternative interpretation that nucleolar RNA synthesis is directly primed by RNA, which is under investigation. The addition of nucleosides strongly suggests that actinomycin does not act primarily by depleting precursor pools (section C). Furthermore, certain antimetabolites (section D) expected to act at the level of pools were more effective on chromosomal than on nucleolar RNA synthesis, and they affected nucleolar synthesis less than actinomycin affected chromosomal synthesis. In contrast to actinomycin the effect of these antimetabolites is rapidly reversed.

If DNA is the primer for the nucleolus the question would arise where in the nucleus is the primer. As shown by light[4] and electron[17] microscope autoradiography, RNA label appears to initiate from the beginning in the nucleolus itself and not in the DNA-containing nucleolus *organizer* of these nuclei; this holds down to a 2 min. pulse and 3 months' exposure of light microscope autoradiographs, which is near the limit of the techniques. Therefore, either the primer is the organizer DNA and the RNA moves and builds up very fast in the nucleolus, or the primer is not that DNA (which is plausible since other nucleoli are free and without organizer)[18]. DNA is not cytochemically detectable in the *nucleolus* itself[4]. Using high doses of thymidine over a larval period representing a ten-fold growth of the nucleolus, no evidence was found for nucleolar DNA replicating in step with chromosomal DNA (section A). In the older stages investigated the maximum possible amount of undetected nucleolar DNA was estimated in the order of 10^{-3} of total chromosomal DNA. An alternative postulate put forward earlier was that *chromosome* DNA in general primes nucleolar RNA[6]. The priming would have to last long to account for the observed autonomy of nucleolar synthesis (*see* below), which has still to be proved. The nature and origin of the primer have then to be elucidated before the operational principle of the nucleolus can be formulated.

The preliminary experiments in section E show nucleolar RNA synthesis while chromosomal synthesis was held inhibited—a condition approaching *in vivo* isolation of nucleolar synthesis. It was previously shown that the nucleolus makes RNA, and does not simply collect it, when after a 2 min. pulse all significant label was nucleolar[1]; this is in fact shown in section D after chromosomal inhibition. The results indicate that the majority of the nucleolar RNA synthesized during incubation is sRNA. It was reported earlier in the present cells[2] as well as in pea[19] that the nucleolus is a centre of methylation of sRNA. Furthermore, biophysical analysis of nucleolar RNA revealed an important pool of sRNA, identified as transfer RNA[19]. The present findings strongly suggest that the nucleolus is not only a site of accumulation and methylation of sRNA, but a centre of sRNA synthesis. The identification of sRNA in the present nucleoli is under way.

The experiments do not exclude the possibility that the nucleolus also synthesizes RNA of higher S values. First, the inhibitor possibly partly inhibits nucleolar synthesis, and it remains to be seen whether different RNA species are preferentially inhibited. Second, the cells studied are at an advanced stage of differentiation, as described in Materials. Work on bacteria[20] and amphibian oocyte nucleoli (Ozban, N., Tandler, C. J. & Sirlin, J. L., unpublished data) suggests that the relative amount of sRNA increases as cell differentiation proceeds. It is, therefore, possible that nucleolar sRNA has become predominant at this stage of differentiation.

Summary

1. Salivary glands of insect larvae are incubated *in vitro* in chemically defined medium.

2. Autoradiography observations on the effect of actinomycin D and proflavin are in line with a primer DNA for nucleolar RNA synthesis but cannot exclude a primer RNA.

3. From the addition of nucleosides a direct effect of actinomycin on nucleolar pools seems improbable.

4. Any DNA present in the nucleolus itself (excluding the organizer) was below detection by incorporation of thymidine over a considerable period of growth of the nucleolus. The evidence for the presence of primer DNA for the nucleolus in the chromosomal organizer versus chromosomes in general is inconclusive.

5. Benzamide and DRB (TRB) inhibited chromosomal more than nucleolar RNA synthesis.

6. Nucleolar RNA synthesized in the presence of DRB was mostly sRNA by biophysical analysis. Some implications of this finding are discussed.

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Metabolic Regulation of Ribonucleic Acid Turnover and Ribonuclease Function

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Several steps of the mechanism of protein biosynthesis have now been delineated. The structural site of protein synthesis is the ribonucleoprotein particle, the ribosome. Amino acids in an activated form are brought by specific carrier RNA molecules (transfer RNA) to the ribosomes, where organization into polypeptide chains is directed by informational or messenger RNA. The messenger RNA is believed to be involved in transcription of the gene message from DNA to the protein-forming systems. The DNA primed RNA polymerase system is now considered to be responsible for the formation of messenger RNA[1].

In view of this intimate relationship between RNA and protein synthesis, ribonucleases may be expected to have a significant function in the control of protein biosynthesis. A wide variety of ribonucleases (RNases) from animals has been described and many of these RNases have been purified and shown to exhibit interesting differences in their structures and specificities. All parts of mammalian cells except nuclei have been shown to contain RNase activity. In rat liver, the largest proportion of RNase activity resides in mitochondria and exhibits *p*H optima of 5·6 and 7·8 (Ref. 2). RNase activity is also associated with supernatant fraction and ribonucleoprotein particles[3]. Subcellular distribution of RNase activity in rat spleen and adipose tissue follows a similar pattern[4,5].

It is of interest to know how the RNase function is regulated inside the living cell. The activity must be controlled in some way in order to prevent breakdown of various cellular ribonucleic acids having defined functions in protein biosynthesis. On the other hand, finer mechanisms must exist to enable latent RNase activity to manifest itself whenever turnover of ribonucleic acids is imperative.

One of the significant findings which may have a bearing on the metabolic regulation of RNase function has been the discovery of RNase inhibitors and there have been a few studies which indicate that these inhibitors may indeed have a pivotal role to play in the regulation of ribonucleic acid turnover. Thus Roth[6] has examined the effect of whole body X-irradiation on the inhibitor levels. The changes in the inhibitor activity have also been studied in different cancerous tissues[7,8] and during liver regeneration following partial hepatectomy[9].

Another interesting fact is the latency of RNase in ribonucleoprotein particles[10–12]. A role in messenger RNA turnover has been attributed to it by Tal and Elson[10]. The picture concerning the functional role of RNase is, however, extremely hazy particularly when we view it against the background of extensive amount of work that has been carried out on the mechanism of action of RNase and the structural aspects of the RNase molecule.

The present paper describes the recognition and characterization of RNases in subcellular fractions from rat adrenals and an inhibitor for RNase in the supernatant fraction which is shown to resemble its liver counterpart in its properties. Difference in the behaviour of acid and alkaline ribonucleases from both rat liver and adrenal mitochondria towards physical treatments is also reported.

In order to gain some insight into the regulatory mechanisms operating in the turnover of ribonucleic acids, we have examined a few simple systems, viz. protein deficiency condition in rats and phosphate starvation of *Escherichia coli* Vitamin B_{12} methionine auxotroph and *Saccharomyces cerevisiae* (a locally isolated strain). In these studies we have also obtained evidence which shows that the proportion of rapidly turned over RNA, under these conditions of restricted precursor supply, is higher than that found under normal conditions of growth.

Experimental

Animal experiments

Male Wistar rats weighing 125-150 g. were divided into two groups and fed the following diets for 3 weeks: (1) *ad lib.* 18% casein diet, and (2) *ad lib.* 0% casein diet.

Fractionation of cell components of rat tissues. Rat liver and adrenals were homogenized with 10 volumes of chilled isotonic sucrose solution. The homogenate was centrifuged at 500 *g* in an International PR-2 refrigerated centrifuge to remove nuclei and unbroken cells. Mitochondria were separated at 5000 *g* for 10 min. These were washed with sucrose solution and recentrifuged under the same conditions. The mitochondrial supernatant was then centrifuged in Spinco model L ultracentrifuge at 10000 *g* for 10 min. to remove light mitochondria. Microsomes and supernatant fractions were obtained by spinning the above supernatant at 105000 *g* for 1 hr. Ribosomes were isolated from the microsomes according to Kirsch *et al.*[13]

Assay of RNase. RNase activity in homogenate and cell fractions was determined by the method of Brody[7]. RNase activity was determined by following the release of acid-soluble nucleotides upon incubation with 1·0 ml. dialysed yeast RNA (2·5 mg./ml.) in a final volume of 3 ml., either at *p*H 5·6 (acetate buffer, 0·02 *M*) or at *p*H 7·8 (tris-HCl buffer, 0·02 *M*).

Mitochondrial pellet isolated in 0·25 *M* sucrose was suspended in chilled glass-distilled water, frozen and thawed twice and used for the RNase assay.

In the case of the supernatant enzyme, preincubation with N-ethylmaleimide ($3 \times 10^{-3} M$ in final volume) was carried out for 30 min. before incubation with substrate. The reaction was stopped by addition of trichloroacetic acid (10%) containing uranyl acetate (0·5%), the precipitate removed by centrifugation, and absorption of the acid-soluble nucleotides determined at 290 mμ. Suitable substrate and enzyme blanks were run simultaneously. RNase activity is expressed as change in optical density units at 290 mμ in 30 min. either per mg. protein nitrogen or per 100 μg. DNA-phosphorus.

Assay of RNase inhibitor. The system for RNase inhibitor assay[14] consisted of 0·2 ml. of supernatant fraction (1: 10), 0·15 ml. of 0·05 *M* EDTA (*p*H 7·8), 0·125 μg. of crystalline pancreatic RNase, 1·0 ml. of veronal, acetate buffer [*p*H 7·8 (0·05 *M*)], and 1·0 ml. of RNA solution (1%), in a final volume of 3 ml. Incubation was carried out for 30 min. at 37°C. The reaction was stopped by adding 3 ml. of precipitating reagent (1 *N* HCl in 76% ethanol containing 0·5% lanthanum chloride) and centrifuged. The supernatant was diluted 1: 50 and absorption at 260 mμ was measured. One unit of inhibitor is equivalent to inhibitor concentration causing 50% inhibition of 0·005 μg. of crystalline RNase.

Chemical determinations. DNA and RNA contents were estimated according to the procedure of Schmidt and Thannhauser[15]. Protein was determined by the method of Lowry *et al.*[16]

Fractionation of liver RNA by column chromatography. RNA isolated from rat livers by the modified phenol extraction procedure of Kirby[17] was adsorbed on a methylated serum albumin-coated kieselguhr column prepared by the procedure of Mandell and Hershey[18] (0·6 mg. RNA per column). The RNA was eluted by a linear gradient of NaCl solution from 0·4-1·0 *M* in 0·04 *M* phosphate buffer (*p*H 6·7). Five ml. fractions were collected and O.D. readings of these were taken at 260 mμ.

Incorporation of ^{32}P into rat liver RNAs. In experiments where ^{32}P incorporation into RNA fractions was examined, orthophosphoric acid-^{32}P in isotonic saline was injected intraperitoneally (100μC/100 g. body weight) and the animals were killed at 15 min., 2 hr and 5 hr thereafter. Two rats were killed at each time interval. These were then treated according to the procedure of Brawerman and coworkers[19] to obtain template RNA, ribosomal RNA and transfer RNA. In this procedure, homogenates of livers in 10% sucrose buffered at pH 7·6 with 0·01 M tris buffer were subjected to fractionation as described above. Nuclear fraction in 0·1 M tris buffer (pH 7·6) was added immediately to an equal volume of chilled 90% aqueous phenol (redistilled). The suspension was stirred for 1 hr at room temperature and the aqueous phase was removed by centrifugation. The interphase was stirred with the phenol phase, equal volume of 0·1 M tris buffer (pH 7·6) was added and the suspension was stirred at room temperature for 1 hr. The aqueous layer was removed and the extraction at pH 7·6 was repeated once. Further extractions were carried out with tris buffer (0·01 M) at pH 8·0 once, at pH 8·5 twice and at pH 9·0 twice. The fractions at pH 9·0 were combined and were designated as template RNA.

The 105000 g supernatant fraction was adjusted with tris buffer (pH 7·6) to 0·1 M and the ribosomes were suspended in 0·1 M tris buffer (pH 9·0) treated with 90% phenol; the aqueous phases were recovered.

RNA from aqueous fractions was precipitated by addition of 0·1 volume of 10% NaCl and 2·5 volumes of ethanol. The precipitates were dissolved in small volumes of ice-cold water and adjusted with NaCl to a concentration of 10%. Storage at 4°C. for 2 days brought about the precipitation of high molecular weight RNA. This was recovered by centrifugation. The RNA preparations were exhaustively dialysed, and subjected to extraction procedure according to Schmidt and Thannhauser[15].

Sensitivity of isolated liver RNA fractions towards pancreatic RNase. The labelled RNAs isolated from livers of rats killed 5 hr after intraperitoneal injection of orthophosphoric acid-^{32}P were examined for their susceptibility towards pancreatic RNase. The incubation system contained: RNA, 50μg. (adjusted by measurement of O.D. at 260 mμ); veronal-acetate buffer of pH 7·8 (0·05 M) and 0·05 μg. of crystalline RNase in a total volume of 3·0 ml. The system was incubated for 30 min. at 37°C. Radioactivity of trichloroacetic acid-soluble nucleotides was determined at 0 and 30 min. The sensitivity of RNA to RNase action is expressed as c.p.m. ^{32}P released per 1000 c.p.m. of RNA-^{32}P.

Base composition analysis of bulk RNA, template RNA and DNA. 20 g. of liver obtained from rats fed 15% casein diet was treated as above to obtain template RNA fraction. The yield was 6 mg. Base compositions of this RNA as well as bulk RNA isolated by the method of Kirby[17] were determined after alkaline hydrolysis and paper chromatography in isobutyric acid—0·5 N NH$_3$ (5: 3, vol./vol.), elution of the spots with phosphate buffer and quantitative ultraviolet spectrophotometry[20]. Composition of rat liver DNA was determined by the procedure of Wyatt[21].

Experiments with Microorganisms

Conditions of growth and phosphate starvation. Conditions of growth and phosphate starvation of *Esch. coli* Vitamin B$_{12}$/methionine auxotroph were reported previously[22]. *Esch. coli* cells previously grown for 18 hr on a medium (medium A; *p*H 8·0) containing per litre, 20 g. glucose, 10 g. peptone, 0·5 g. sodium citrate, 0·1 g. $MgSO_4.7H_2O$, 0·25 g.K_2HPO_4 and 0·036 g. KH_2PO_4, were starved of phosphate by incubation in a medium (*p*H 8·0) containing per litre, 20 g. glucose, 4·0 g. L-asparagine, 0·1 g. L-arginine, 0·1 g. glutamic acid, 0·1 g. glycine, 0·1 g. L-histidine, 0·1 g. $MgSO_4.7H_2O$, 0·5 g. sodium citrate, 12·0 g. tris-(hydroxymethyl)-aminomethane and 50 mg. DL-methionine. Initial density of culture prior to phosphate starvation was 5·2 mg. dry weight per ml.

Conditions of growth and phosphate starvation of *S. cerevisiae* were essentially as reported by Pradhan *et al.*[23] Cells were grown aerobically for 18 hr in a medium containing per litre, glucose 40 g., bactopeptone 10 g., yeast extract 1 g. and KH_2PO_4 1 g. Phosphate starvation was carried out in a medium (—P) containing per litre, glucose 40 g., mixture of amino acids simulating composition of yeast protein[24] equivalent to nitrogen 0·8 g., $MgSO_4$ 0·09 g., Na_2SO_4 0·13 g., and buffered with 0·02 *M* citrate buffer. The initial density of the culture prior to phosphate starvation was adjusted to 0·6 g. dry weight per litre.

Assay of RNase activity. RNase activity of washed cells was measured by the method of Brody[7] as described under animal experiments using cells equivalent to 9-10 mg. protein. Units of enzyme activity are expressed as change in optical density at 260 mμ.

Assay of acid and alkaline phosphatases (phosphomonoesterases). Acid phosphatase in *S. cerevisiae* was measured by the method of von Hofsten[25]. The assay mixture for acid phosphatase consisted of cell suspension 0·1 ml. (8-10 mg. protein), *p*-nitrophenylphosphate (Sigma) (5 mμ moles), and acetate buffer (*p*H 4·5) added to a final volume of 2·0 ml. Incubation was carried out for 10 min. at 30°C. Two ml. of 0·1 *N* NaOH was then added to stop the reaction and develop the colour which was measured at 400 mμ. Units of enzyme activities are expressed as mμ moles of *p*-nitrophenol liberated under the above conditions.

Alkaline phosphatase was estimated as reported previously[22]. The assay mixture consisted of 0·1 ml. of cell suspension, 1 ml. of 0·01 *M* sodium phenyl phosphate, and 0·1 *M* glycine-sodium hydroxide buffer in a final volume of 4·0 ml.; incubation was carried out for 1 hr at 30°C. Units of enzyme activities are expressed as mμ moles of phenol liberated under the above conditions.

Pulse labelling of *Saccharomyces cerevisiae*. Pulse experiments were carried out on (1) *Saccharomyces cerevisiae* cells growing logarithmically on a medium containing per litre, bactopeptone 10 g., yeast extract 1·0 g. and glucose 40 g., and (2) *Saccharomyces cerevisiae* cells, phosphate starved for 16 hr on the medium (—P). It has been reported earlier[23] that although there is a net breakdown of RNA, a turnover of RNA takes place at this stage of phosphate starvation.

Twenty ml. of the culture (7·2 mg. dry weight per ml.) were given a 3-min. pulse of 100 μC orthophosphoric acid-^{32}P and RNA was immediately isolated and purified; the nucleotide composition of pulse-RNA was determined according to the procedure laid down by Spiegelman and coworkers[26]. ^{32}P given was carrier-free and the amount added to the phosphate starved culture was so small that there could not be any alteration in the phosphate starved condition.

Measurement of radioactivity. The radioactivity determinations of RNA fractions and nucleotides were carried out in a Tracerlab SC-16 windowless gas-flow counter in conjunction with a Tracerlab SC-51 autoscaler.

Materials. Crystalline pancreatic RNase was obtained from Worthington Biochemicals, Freehold, New Jersey. RNA and DNA were products of Sigma Chemicals. Calcium phosphate was prepared by the method of Tiselius *et al.*[27] ^{32}P-orthophosphoric acid (carrier-free) was obtained from the Atomic Energy Establishment, Trombay, Bombay, India.

RESULTS

Subcellular distribution of RNases

Table 1 summarizes the data obtained on the subcellular distribution of RNases in rat liver and adrenals. The mitochondrial RNases in both tissues exhibit *p*H optima of 5.6 and 7·8. Hence the free and total RNase activity was measured at these *p*H values. A 1 : 10 suspension of mitochondria

TABLE 1—DISTRIBUTION OF RIBONUCLEASE ACTIVITY IN SUBCELLULAR FRACTIONS FROM RAT LIVER AND ADRENALS

[RNase activity determinations were carried out by following the release of acid-soluble nucleotides upon incubation of 1 : 10 homogenate or equivalent cell fraction with dialysed yeast RNA (2·5 mg./ml.). Total RNase activity of mitochondria at *p*H 5·6 and 7·8 was determined either after freezing and thawing a 1 : 10 suspension of mitochondria distilled water* or after treatment with triton X-100 (0·1%, vol./vol.)**. The supernatant activity was assayed with and without preincubation with N-ethylmaleimide (3×10^{-3} *M* final concentration). The activities are expressed as increase in absorbancy at 290 mμ (A_{290}) in 30 min./50 mg. tissue or equivalent cell fraction]

Tissue	Ribonuclease activity (A_{290})						
	Homogenate	Mitochondria				Supernatant	
		Free activity		Total activity		−N-ethyl maleimide	+N-ethyl maleimide
		*p*H 5·6	*p*H 7·8	*p*H 5·6	*p*H 7·8	*p*H 7·8	*p*H 7·8
Adrenals	0·375	0·100	0·108	*0·398	*0·108	0·095	0·280
				**0·412	**0·112		
Liver	0·250	0·124	0·124	*0·342	*0·118	0·080	0·220
				**0·400	**0·121		

in 0·25 *M* sucrose was used in the assay system for determination of free RNase activity. Total mitochondrial RNase activity was assayed after subjecting the mitochondrial suspension in water (1 : 10) to freezing and thawing at least twice, or by treatment of a 1 : 10 suspension of mitochondria in 0·25 *M* sucrose with triton X-100 (0·1%, vol./vol.). There was three to four-fold increase in total acid RNase activity (*p*H 5·6), whereas alkaline RNase activity (*p*H 7·8) remained unaffected. The stimulation of acid RNase activity after these treatments suggests that this enzyme may be located in the membrane portion of mitochondria. Similar stimulation by treatment with detergents has been reported for glucose-6-phosphatase of rat liver microsomes[28].

Supernatant RNase activity (*p*H 7·8) is increased by treatment with N-ethylmaleimide which suggests the presence of an inhibitor having free —SH groups.

Characterization of adrenal RNase inhibitor

An inhibitor of RNase was found to be present in rat adrenals in at least the same intensity as in rat liver. It was, therefore, thought of interest to study the characteristics of this inhibitor and to ascertain whether these characteristics are similar to those reported for liver RNase inhibitor.

Time course study of the action of N-ethylmaleimide on the adrenal inhibitor and on the inhibitor in the presence of crystalline pancreatic RNase was done. The conditions of the experiment and the results are given in Fig. 1. The RNase activity in the adrenal supernatant is very low; on addition of N-ethylmaleimide a three-fold increase in activity is observable, indicating the presence of a bound form of RNase in the supernatant fraction which is released upon inactivation of the inhibitor by —SH binding reagents like N-ethylmaleimide. However, the supernatant fraction is not saturated with respect to RNase, since considerable amount of added crystalline enzyme may be inactivated (Curve 3) by addition of supernatant fraction. Reversal of this inhibition by prior treatment of adrenal supernatant with N-ethylmaleimide is shown by Curve 5.

Preliminary studies on the purification of the inhibitor compound by combined ammonium sulphate precipitation and calcium phosphate gel treatment are summarized in Table 2.

The inhibitor activity is not lost upon dialysis of the supernatant fraction or the partially purified inhibitor preparation. Upon heating at 55°C. for 5 min., 90% of the activity is destroyed. These results are indicative of the protein nature of the inhibitor. The characteristics, like presence of —SH groups, heat liability and non-dialysability of adrenal inhibitor are similar to those reported for rat liver inhibitor[2].

Levels of RNase and RNase inhibitor during protein fasting

RNase activities of sub-cellular fractions in normal and protein-fasted

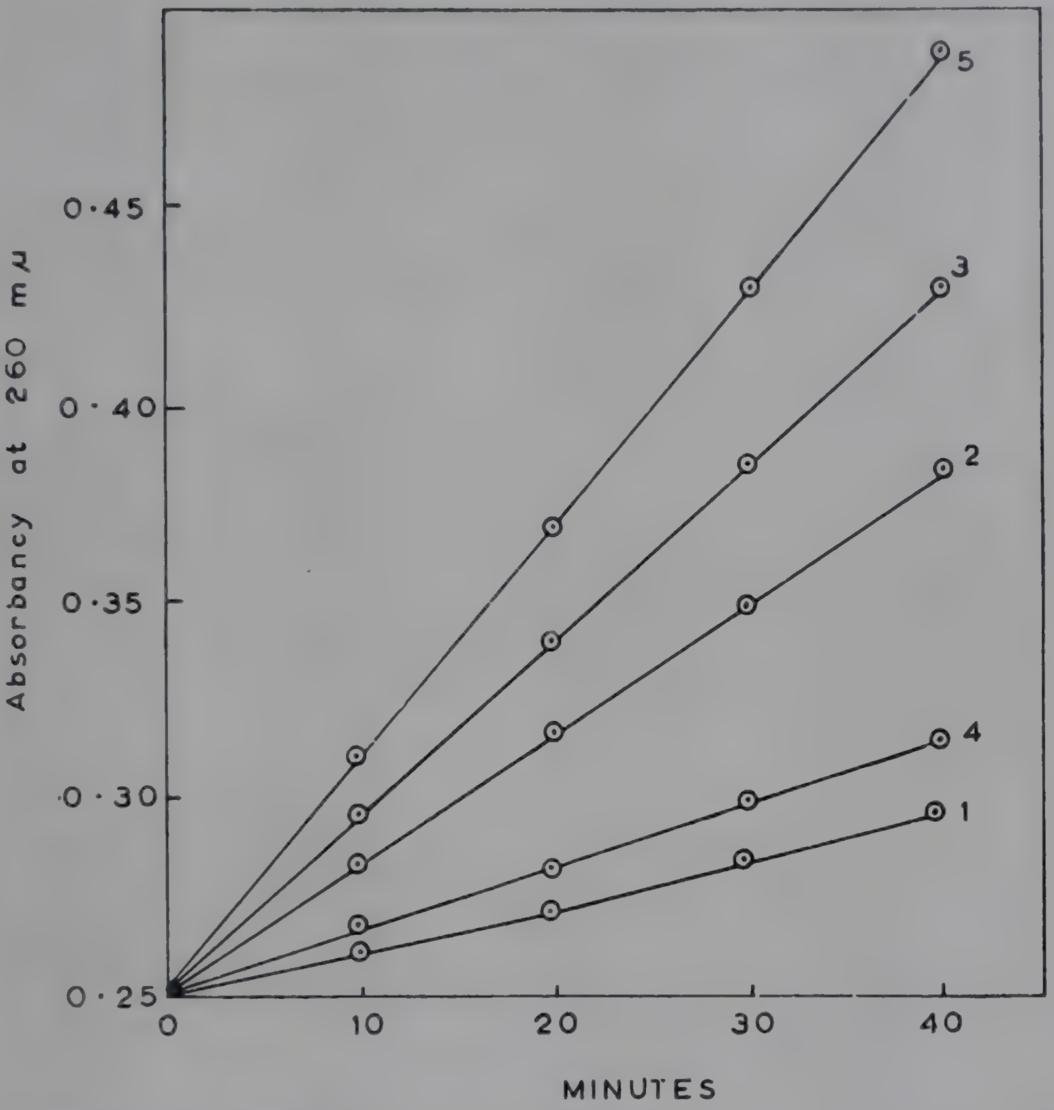

Fig. 1—The effect of N-ethylmaleimide on the inhibition of crystalline pancreatic RNase in adrenal supernatant: The assay system consisted of 10·0 ml. of veronal acetate buffer of *p*H 7·8 (0·05 *M*), 10·0 ml. of yeast RNA (1%), 1·0 μg. of crystalline RNase, 4·0 ml. of supernatant (1:10), and N-ethylmaleimide (3×10^{-3} *M* final concentration), to give a total volume of 25 ml. 4 ml. aliquots were added at 0, 10, 20, 30 and 40 min. intervals to 4·0 ml. of acid-alcohol. The supernatant was diluted 1:50 and readings were taken at 260 mμ. Curve 1 is the activity of adrenal supernatant RNase alone. Curve 2 is the activity of supernatant fraction preincubated with N-ethylmaleimide at 37°C. for 30 min. Activity of crystalline RNase and its inhibition by adrenal supernatant are represented by graphs 3 and 4 respectively. Curve 5 shows the reversal of this inhibition (Curve 4) by N-ethylmaleimide

rat livers are shown in Table 3. The levels of alkaline RNase activity of homogenate and supernatant expressed either in terms of protein or DNA-phosphorus show an increase in protein-fasted livers compared to controls. The level of acid RNase activity (*p*H 5·6) which is exclusively located in the mitochondria and hence assayed in this fraction, is apparently unaffected. Since the supernatant fraction contains considerable amount of activity (*p*H 7·8) in combination with the inhibitor compound[29], the enzyme activity is measured after treatment of the supernatant with N-ethylmaleimide (3×10^{-3} *M* final concentration) which inactivates the inhibitor by binding

TABLE 2—PURIFICATION OF RAT ADRENAL INHIBITOR IN SUPERNATANT FRACTION

[Supernatant fraction (1 : 3) was treated with solid ammonium sulphate to 35% saturation. The precipitate was removed by centrifugation at 60000 *g* for 20 min. and discarded. Ammonium sulphate was added to the supernatant to 55% saturation and centrifuged at 60000 *g* for 20 min. The pellet was suspended in cold distilled water, treated with calcium phosphate gel and eluted with phosphate buffer *p*H 7·3 (0·2 *M*). One unit of inhibitor is equivalent to the inhibitor concentration causing 50% inhibition of 0·005 μg. of crystalline pancreatic RNase]

Fraction	Inhibitor activity units	Protein mg.	Specific activity
Original supernatant	28·0	3·168	8·8
55% Ammonium sulphate precipitate	28·0	0·92	30·4
Eluate from calcium phosphate gel	42·0	0·4095	102·4

TABLE 3—EFFECT OF PROTEIN DEPLETION ON RNASE ACTIVITY IN RAT LIVER CELL FRACTIONS

[Subcellular fractions from normal and protein fasted rat livers were isolated as described in the text. RNase activity was determined by following the release of acid-soluble nucleotides by 0·5 ml. of 1 : 10 homogenate or equivalent cell fraction from 1·0 ml. of dialysed yeast RNA (2·5 mg./ml.) in a final volume of 3·0 ml., during 30 min. incubation period at 37°C. The reaction was stopped by addition of 3·0 ml. TCA (10%) containing uranylacetate (0·5%); the increase in absorbancy of the supernatant at 290 mμ (A_{290}) was determined after centrifugation. The results are given as mean of six individual observations ±S.E.M.]

Fraction	RNA concentration in mg./g. liver		Ribonuclease activity (A_{290})			
			per mg. protein		per 10 μg. DNA-P	
	Protein deficient	Control	Protein deficient	Control	Protein deficient	Control
Homogenate	0· 52± 0·018	0· 76± 0·024	0·065 ±0·002	0·026 ±0·001	3·35 ±0·136	2·144 ±0·129
Mitochondria (*p*H 7·8)			0· 17 ±0·013	0·112 ±0·012		
Mitochondria (*p*H 5·6)			0·347 ±0·012	0·367 ±0·018		
Supernatant			0·083 ±0·002	0·057 ±0·003	2· 26 ±0·145	1·599 ±0·129

the free sulphydryl groups. Under the conditions of assay, there is a 20-25% increase in the supernatant enzyme activity in protein-fasted livers. In mitochondria, RNase activity is expressed on the basis of protein only since there are losses during isolation of pure mitochondrial fraction and hence quantitative yields are not obtained. Calculation of results in terms of DNA-phosphorus content which is proportional to cell number renders possible evaluation and comparison of enzymatic activities in normal and protein-depleted animals against a standard of reference unaffected by the diet.

The inhibitor registers a fall in activity in protein-depleted animals (Table 4). The inhibitor level assayed in these experiments is the free inhibitor which is in excess over the alkaline RNase of the supernatant. It appears that the increase in RNase activity observed by us and by others[30] is essentially the result of lowering of inhibitor protein levels in the induced stress condition and possibly provides a simple device for the regulation of intracellular ribonuclease activities.

RNA profiles and synthetic patterns during protein-fasting

In view of the increased ribonuclease levels in protein depleted conditions it was of interest to examine synthesis of various cellular RNAs under these conditions. Methylated albumin-coated kieselguhr column chromatography[18] worked out well for separation of RNA isolated from total liver by phenol procedure[17]. With this method we also obtained results which showed that profiles of RNA isolated from protein-fasted rat livers were somewhat different from those of normal rat livers. The proportion of low molecular weight RNA which is eluted earlier is higher in protein-fasted rats than in normal ones. On the other hand, proportion of high molecular weight RNA which is eluted in the later fractions is lowered in protein deficiency (Fig. 2). However, rapidly labelled RNA could not be separated very effectively from the bulk RNA and hence a different method was sought to examine the kinetics of synthesis of liver RNAs.

Our earlier studies had indicated that RNA from nuclear fraction of rat liver acquires a much higher specific activity one hour following ^{32}P injection, compared to soluble or ribosomal fraction. These considerations prompted us to adopt a method recently reported by Brawerman and coworkers[19]. In this procedure, nuclear RNA fractions are obtained by phenol extraction at pH 7·6, followed by extraction of the interphase at pH 8·5 and 9·0. Brawerman and coworkers[19] designate pH 9·0 fraction as the template RNA fraction since it showed stimulation of incorporation of leucine into protein by a cell-free preparation of *Esch. coli*[31] and in this respect was 100 times more effective than ribosomal RNA.

The results of incorporation experiments with ^{32}P are shown in Table 5. The template RNA (pH 9·0 fraction of nuclear RNA) shows the highest specific activity at 15 min. compared to transfer RNA or ribosomal RNA. After longer exposure times (2-5 hr), the difference in the labelling pattern is less marked. It will be noted from Table 6 that the rate of synthesis of RNA is not impaired in protein starvation and the low cellular RNA content (Table 3) is mainly due to excessive elaboration of ribonuclease which apparently provides free precursor nucleotide pool for new RNA formation. In fact, our data reveal predominantly higher proportions of rapidly labelled template RNA fractions in the protein-fasted conditions.

The nucleotide composition of template RNA is shown in Table 7. This fraction somewhat simulates DNA in nucleotide composition but

TABLE 4—Effect of Protein Depletion on Levels of RNase Inhibitor in Rat Liver Supernatant

[The assay system of RNase inhibitor consisted of 0·2 ml. of supernatant fraction (1 : 10), 0·15 ml. of 0·05 *M* EDTA (*p*H 7·8), 0·5 ml. of solution of crystalline enzyme (0·125 μg.), 1·0 ml. of veronal acetate buffer of *p*H 7·8 (0·05 *M*), and 1 ml. of RNA solution (1%), in a final volume of 3·0 ml. After incubation for 30 min. at 37°C. the reaction was stopped by acid-alcohol. The supernatant was diluted 1 : 50 and absorption at 260 mμ was measured. The results are means of six individual experiments ± S.E.M. Expression of inhibitor activity is as described under Table 2]

Animals	Units per mg. protein	Units per 100 μg. DNA-P
Protein deficient	8·31±0·28	252·3±23·2
Controls	13·66±0·12	510·3±15·4

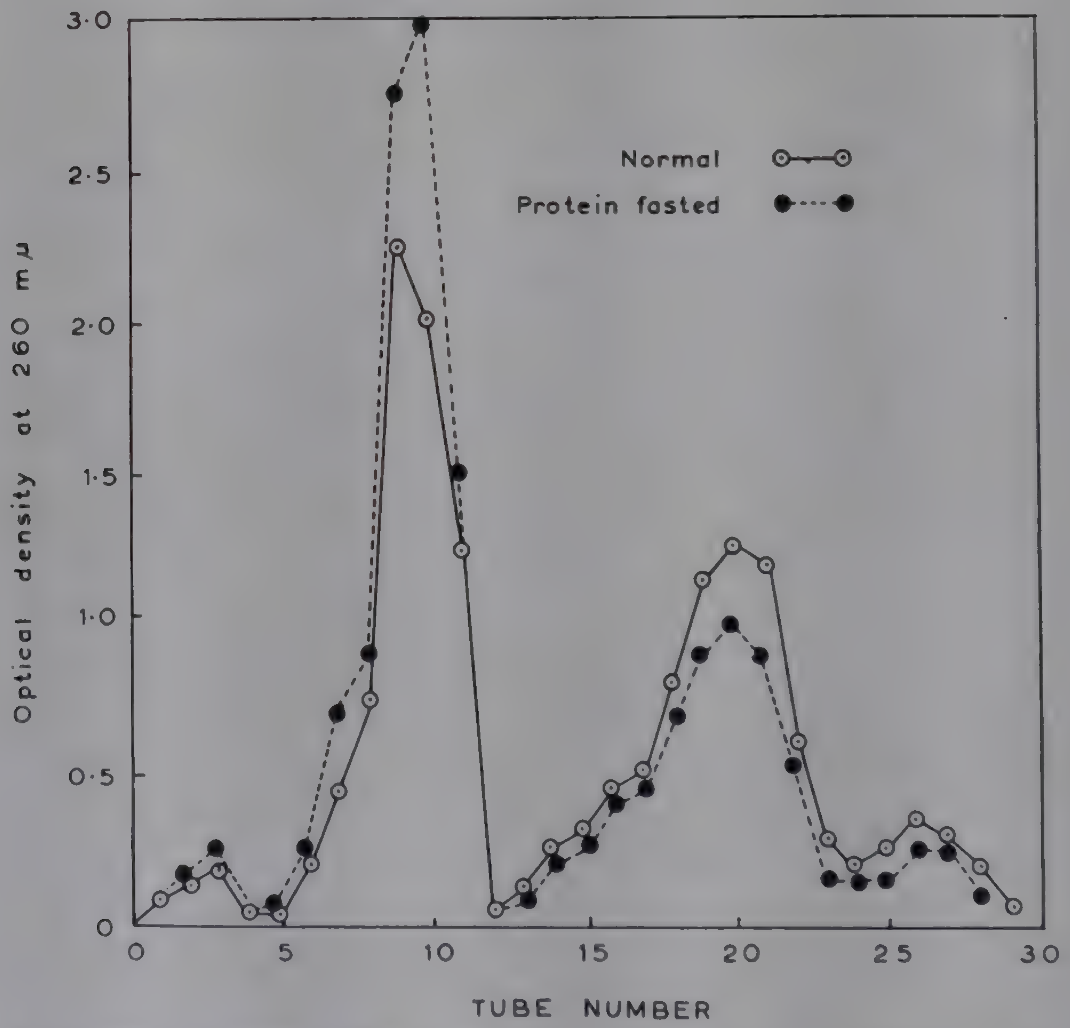

Fig. 2—Elution patterns of RNAs from livers of normal and protein-fasted rats: RNA isolated from rat livers by the modified extraction procedure of Kirby[16] was adsorbed on a methylated serum albumin-coated kiesulguhr column prepared by the procedure of Mandell and Hershey[17] (0·6 mg. RNA per column). The RNA was eluted by a linear gradient of NaCl solution from 0·4-1·0 *M* in 0·04 *M* phosphate buffer (*p*H 6·7). 5 ml. fractions were collected and optical density readings of these were taken at 260 mμ. ⊙—⊙, Liver RNA profiles of normal rat. ●—●, Liver RNA profiles of protein tested rats]

TABLE 5—KINETICS OF INCORPORATION OF ^{32}P INTO LIVER RIBONUCLEIC ACIDS OF NORMAL RATS

[Animals were injected intraperitoneally with orthophosphoric acid-^{32}P (100 μg./100 g. body weight) in isotonic saline and sacrificed at 15 min. 2 hr and 5 hr after the injections. The RNA fractions were obtained from the subcellular fractions by the procedure described in the text. These were further subjected to extraction procedure of Schmidt and Thannhauser[15] and radioactivity was determined in alkali-soluble RNA components]

Time	Rat no.	Template RNA (*p*H 9·0 nuclear fraction) (c.p.m./mg. RNA)	Transfer RNA (c.p.m./mg. RNA)	Ribosomal RNA (c.p.m./mg. RNA)
15 min.	1	732	210	252
	2	782	276	299
2 hr	3	1200	750	762
	4	1210	733	760
5 hr	5	8000	6300	7250
	6	9000	7600	8250

TABLE 6—KINETICS OF INCORPORATION OF ^{32}P INTO LIVER RIBONUCLEIC ACIDS OF PROTEIN-FASTED RATS

[Animals, protein-fasted for 3 weeks, were injected intraperitoneally with orthophosphoric acid-^{32}P (100 μC/100 g. body weight) in isotonic saline and sacrificed at 15 min., 2 hr and 5 hr after the injections. For other details, see Table 5]

Time	Rat no.	Template RNA (*p*H 9·0 nuclear fraction) (c.p.m./mg. RNA)	Transfer RNA (c.p.m./mg. RNA)	Ribosomal RNA (c.p.m./mg. RNA)
15 minutes	7	846	185	205
	8	833	200	248
2 hr	9	1200	633	680
	10	1250	640	632
5 hr	11	8500	5122	5650
	12	8700	5438	6100

TABLE 7—NUCLEOTIDE COMPOSITION OF TEMPLATE RNA FROM RAT LIVER NUCLEI

[RNA was hydrolysed with alkali and the nucleotide composition determined by the method of Brawerman and Chargaff[20]. DNA composition was determined by the method of Wyatt[21]. Nucleotide composition data are expressed as moles per 100 moles of total nucleotides. A, G, C, U & T refer to adenylic acid, guanylic acid, cytidylic acid, uridylic acid and thymidylic acid. For details, see Experimental]

Nucleotide component	Template RNA	DNA	Bulk RNA
	(moles/100 moles of total nucleotides)		
A	25·6	28·6	19·0
G	28·0	21·5	33·2
C	24·8	21·4	27·5
U (T)	21·6	28·5	20·3

TABLE 8 –SENSITIVITY OF ISOLATED LIVER RNA FRACTIONS TOWARDS PANCREATIC RNASE

[Rats were killed 5 hr after intraperitoneal injection of orthophosphoric acid-^{32}P (100 μC/100 g. body weight). Template RNA, transfer RNA and ribosomal RNA were isolated as described under Experimental. RNA fractions were incubated for 30 min. at 37°C. with pancreatic crystalline RNase as follows: RNA (50 μg., adjusted by measurement of O.D. at 260 mμ), veronal acetate buffer of *p*H 7·8 (0·05 *M*), and 0·05 μg. of crystalline RNase, in a total volume of 3·0 ml. Radioactivity of TCA-soluble nucleotides was determined at 0 and 30 min. The sensitivity of RNA to RNase action is expressed as c.p.m. ^{32}P released/1000 c.p.m. of RNA-^{32}P]

Fraction	RNA (50 μg.) (total c.p.m.)	^{32}P release (c.p.m.)	^{32}P released / 1000 c.p.m. of RNA
Template RNA	300	120	400
Transfer RNA	280	106	380
Ribosomal RNA	350	112	320

differs appreciably from that of bulk RNA. It is quite probable that the template RNA fraction still contains substantial amount of ribosomal RNA. We are attempting to purify this fraction further.

No attempt has been made to separate template RNA from the ribosomal and transfer RNA fractions. Hoagland[32] has presented evidence for the occurrence in rat liver supernatant of a template RNA containing fraction having certain properties in common with bacterial messenger RNA. It may well be that the early appearance of radioactivity in ribosomal or soluble RNA is partly due to association of template RNA with these fractions. Experiments are in progress to study the biological functions of these isolated fractions by examining stimulation of amino acid incorporation into protein by preincubated *Esch. coli* ribosomes[31].

The action of crystalline ribonuclease towards the template RNA fraction is compared with the rates of degradation of ribosomal and transfer RNAs. The results do not show appreciable differences (Table 8).

Phosphate starvation in *Saccharomyces cerevisiae* and *Escherichia coli*

Experiments were also conducted on ribonuclease elaboration and RNA turnover in *Esch. coli* vitamin B_{12}/methionine auxotroph and *Saccharomyces cerevisiae*. It was reported earlier that induction of alkaline phosphatase during phosphate starvation of *Esch. coli* vitamin B_{12}/methionine auxotroph is a phenomenon of *de novo* synthesis of proteins[22]. It will be seen from Table 9 that during phosphate starvation of this strain, protein and DNA contents are increased, whereas there is no change in RNA content. There is a significant increase in ribonuclease activity. If in addition to phosphate deprivation, methionine is withheld from the medium, alkaline phosphatase formation is completely arrested but increase in ribonuclease still continues (Fig. 3).

Under the condition of phosphate starvation of *Saccharomyces cerevisiae*, both acid phosphatase and ribonuclease activities record an increase (Table 10). If in addition to phosphate, nitrogen source is withheld from the medium, acid phosphatase increase is arrested whereas increase in ribonuclease is unaffected (Fig. 4). These results indicate that increase in ribonuclease activity in these strains may not be associated with *de novo* synthesis of proteins.

At 16 hr of phosphate starvation there is a net breakdown of RNA (Table 10), although increment in protein still continues. However, it has been reported earlier that a turnover of RNA takes place at this stage[23]. Table 11 shows the nucleotide composition of pulse-labelled RNA under normal logarithmic growth and under conditions of phosphate starvation for 16 hr. It will be seen that pulse-labelled RNA during phosphate starvation simulates very well DNA in nucleotide composition whereas that obtained during normal conditions has a nucleotide composition simulating

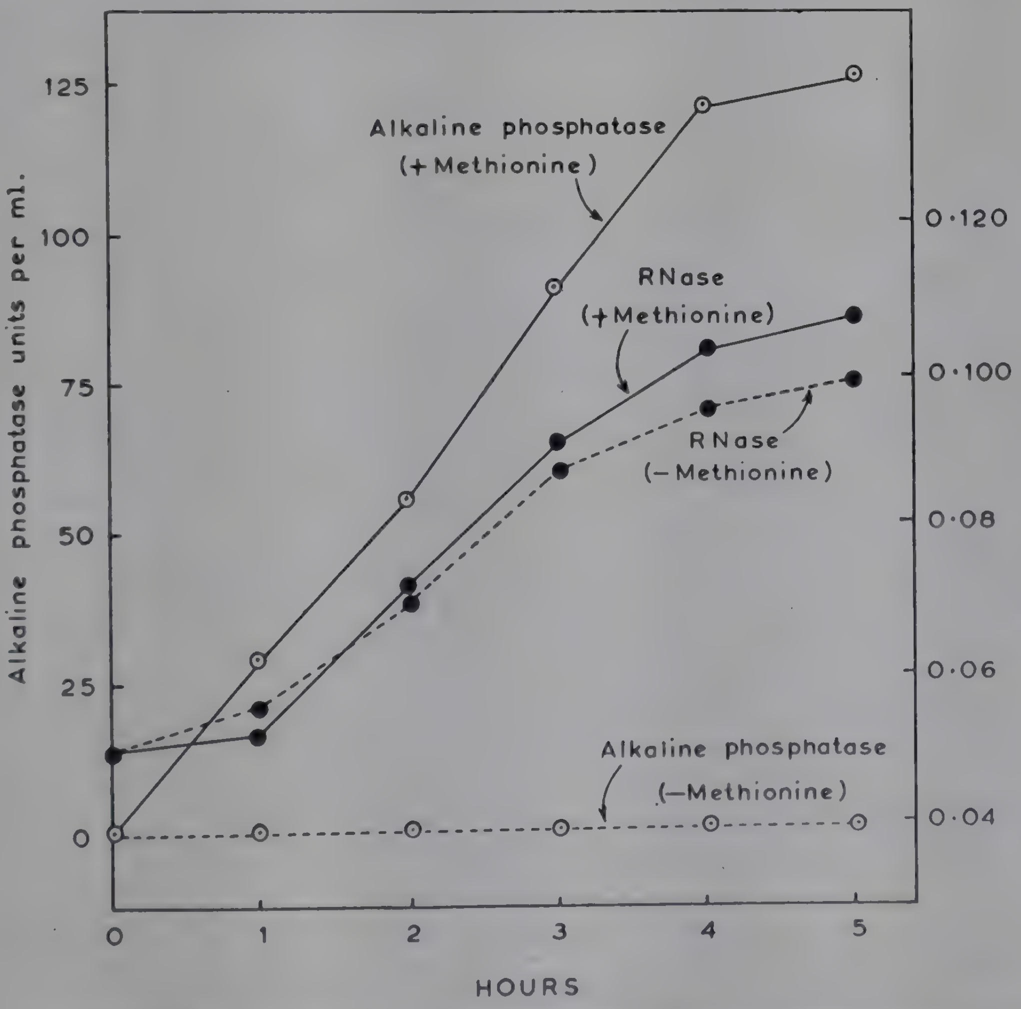

FIG. 3—APPEARANCE OF ALKALINE PHOSPHATASE AND RNASE ACTIVITIES DURING PHOSPHATE STARVATION OF *Esch. coli* B_{12}/METHIONINE AUXOTROPH: EFFECT OF METHIONINE IN THE MEDIUM: *Esch. coli* cells, grown aerobically for 18 hr on medium B containing 8·25 g. K_2HPO_4 per litre, were harvested, washed and then incubated in fresh medium B with and without methionine. Aliquots were removed at one hour intervals up to 5 hr and alkaline phosphatase and RNase activities were assayed in washed cells. Details of composition of media and enzyme assays are given under Experimental

TABLE 9—Appearance of Alkaline Phosphatase and Ribonuclease Activities During Phosphate Starvation of *Esch. coli* B_{12}/Methionine Auxotroph

[*Esch. coli* cells grown aerobically for 18 hr on medium A, were starved of phosphate by incubation in medium B. Initial density of culture prior to phosphate starvation was 5·2 mg. dry weight / ml. Ten ml. aliquots of cell suspensions were removed at the intervals indicated, for chemical determinations and enzyme assays. Details of composition of media, protein, RNA and DNA determinations, and conditions of enzyme assays are given under Experimental. Units of RNase activity are expressed as change in O.D. at 260 mμ. Substrate was dialysed yeast RNA (2·5 mg. / ml.). Incubation was carried at *p*H 7·8 for 30 min. Units of alkaline phosphatase are mμmoles of phenol liberated from 0·0025 *M* sodium phenylphosphate at 30°C. for 1 hr]

Time of incubation hr	Protein μg.	RNA μg./ml.	DNA μg.	Alkaline phosphatase units/ml.	Ribonuclease units/ml.
0	3·2	1·19	0·266	0	0·05
1	3·6	1·20	0·276	36	0·06
2	4·0	1·20	0·300	60	0·08
3	4·4	1·19	0·330	88	0·10
4	4·8	1·19	0·360	125	0·108
5	4·8	1·19	0·365	125	0·108

TABLE 10—Appearance of Acid Phosphatase and Ribonuclease During Phosphate Starvation of *Saccharomyces cerevisiae*

[*S. cerevisiae* cells grown aerobically for 18 hr on bactopeptone-yeast extract medium were starved of phosphate by incubation in phosphate-free medium (synthetic medium containing amino acids). Initial density of culture prior to phosphate starvation was 0·6 mg. dry weight per ml. Fifty ml. aliquots of cell suspensions were removed at the intervals indicated for chemical determinations and enzyme assays. Details of composition of media, protein and RNA determinations and conditions of enzyme assays are given under Experimental. Units of RNase activity are expressed as change in O.D. at 260 mμ. Substrate was dialysed yeast RNA (2·5 mg./ml.). Incubation was carried out at *p*H 7·8 for 30 min. Units of acid phosphatase activity are expressed as mμmoles of *p*-nitrophenol liberated from *p*-nitrophenylphosphate (5 μmoles) at 30°C. in 10 min.]

Time of incubation hr	Protein μg.	RNA μg.	Acid phosphatase units/ml.	Ribonuclease units/ml.
0	585	117	19	0·00
4	655	131	32	0·038
8	763	165	68	0·055
12	1040	176	102	0·082
16	1073	156	154	0·108
20	1092	130	200	0·154

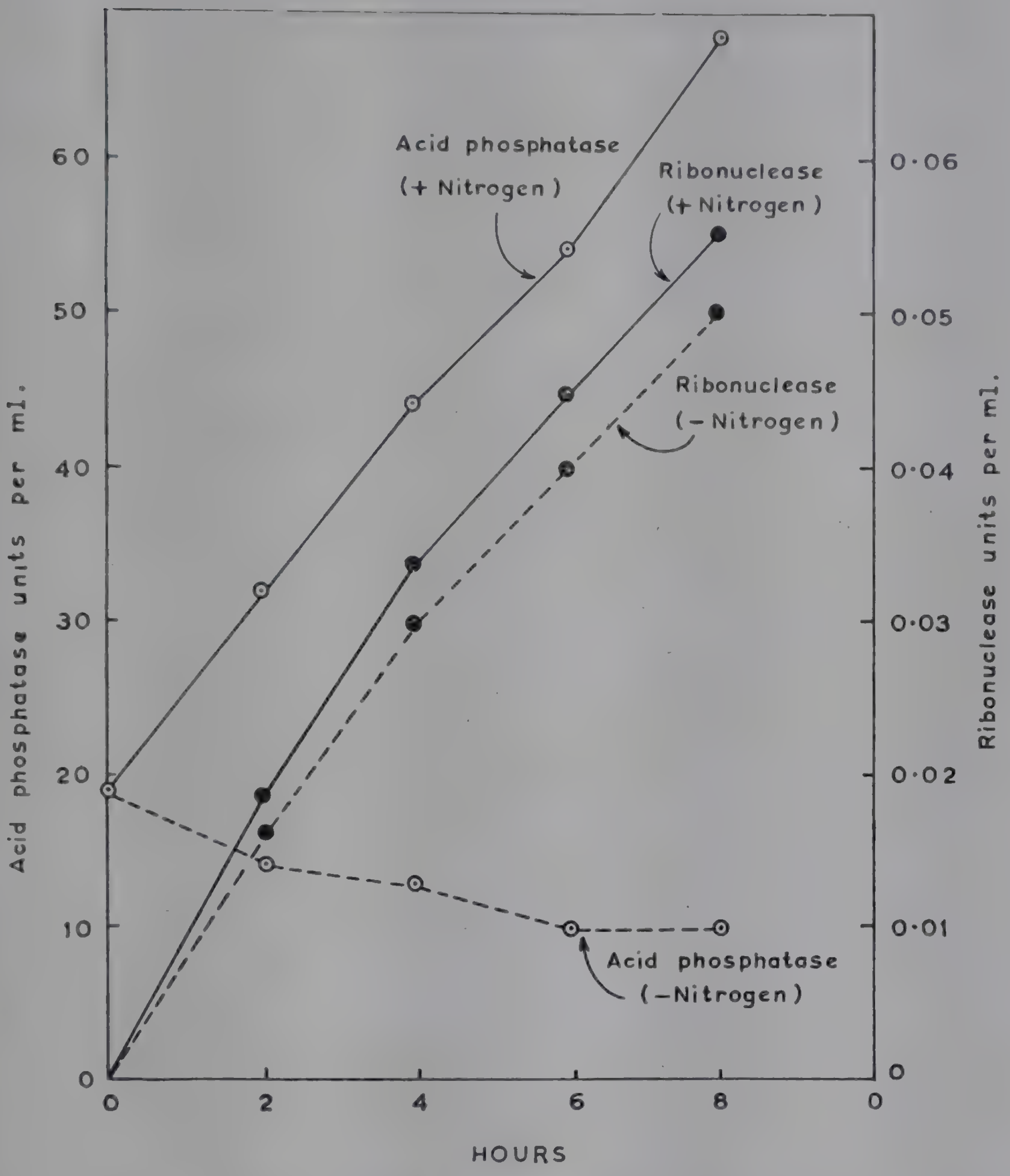

Fig. 4—Appearance of acid phosphatase and RNase during phosphate starvation of *S. cerevisiae:* effect of nitrogen in the medium: *S. cerevisiae* cells, grown aerobically for 18 hr on bactopeptone-yeast extract medium, were harvested, washed and then incubated in the synthetic phosphate-free medium with and without nitrogen source. Fifty ml. aliquots were removed at one hr intervals up to 8 hr of phosphate starvation and acid phosphatase and RNase activities were assayed in washed cells. Details of composition of media and enzyme assays are as given in Experimental. For expression of enzyme units, see Table 10

that of bulk RNA. It is apparent from this experiment that there is a preferential synthesis of messenger RNA under the conditions of phosphate starvation.

Discussion

There are a number of reports in the literature which point to an inverse relationship between ribonuclease activity and nitrogen intake[30]. We have also observed this relationship in the present investigation. Alkaline ribonuclease activity is significantly enhanced in protein fasting whereas

TABLE 11—BASE RATIOS OF PULSE—RNA DURING PHOSPHATE STARVATION OF *Saccharomyces cerevisiae*

[Cells growing in logarithmic phase on bactopeptone-yeast extract medium (see Experimental) and cells which were phosphate starved for 16 hr as described in Table 10, were given ^{32}P pulse for 3 minutes (100 μC per 20·0 ml. culture). The labelled cells were immediately chilled, harvested and RNA was isolated, purified and nucleotide composition determined as described in the text. Total RNA and DNA compositions are given for purposes of comparison. C, A, U, G, & T refer to cytidylic acid, adenylic acid, uridylic acid, guanylic acid and thymidylic acid, respectively]

Nucleotide component	C	A	U (T)	G
	(moles/100 moles of nucleotide)			
Normal	21·4	23·9	31·5	23·2
Phosphate starvation (16 hr)	18·0	31·0	29·2	21·8
DNA	17·1	31·0	32·9	19
Total RNA	21·0	22·0	31·0	26·0

acid ribonuclease activity is practically unaffected. The results of the present investigation on the levels of alkaline ribonuclease inhibitor during protein fasting indicate that elaboration of ribonuclease activity is intimately integrated to the changes in ribonuclease inhibitor levels.

Preliminary work reported in this paper on the purification of the adrenal ribonuclease inhibitor and the demonstration of free reactive sulphydryl groups in its molecule support the contention that the inhibitor is protein in nature[33]. It has now been recognized that proteins differ in their stability towards degradation under stress conditions[34]. The catabolic enzymes like proteases and ribonucleases seem to be quite stable under severe depletion of nitrogen, whereas ribonuclease inhibitor appears to be labile. Since in protein fasting there is a significant lowering of inhibitor level, one may conclude that the increase in alkaline ribonuclease activity is mainly caused by release of ribonuclease from ribonuclease-inhibitor complex and not by a mechanism which would permit preferential synthesis of ribonuclease.

Our data on phosphate starvation of *Esch. coli* and *Saccharomyces cerevisiae* also support the idea that elaboration of ribonuclease activity could be a phenomenon of activation of a latent enzyme rather than *de novo* synthesis of enzyme protein. However, mechanism of activation may differ from that described above. Recently, reports have appeared which show that ribonucleases of *Esch. coli* and yeast are entirely located as latent enzymes in the ribosomes[10,12]. Elaboration of ribonuclease during phosphate starvation may be due to dissociation of ribonucleoprotein particles. Horiuchi[35] has reported similar dissociation of 40s and 29s particles in phosphate deficient *Esch. coli* cells. Although the latency of ribonuclease in actively protein-synthesizing systems appears to be essential, it should be noted that these ribonucleases could also participate in the pliable systems which control biosynthesis of specific proteins during changes in environmental conditions. It has been suggested[10] that the messenger RNA may be inactivated by the ribosomal ribonuclease, thus destroying the ability of the RNA to become attached to ribosomes. Once released from the ribosomes, the messenger RNA might then be degraded by the non-ribosomal nucleases of the cell, such as polynucleotide phosphorylase.

Our data on ^{32}P incorporation in protein-fasted rats clearly indicate that elaboration of ribonuclease activity does not interfere with the synthesis of RNA. The alteration in the synthetic pattern is largely due to preferential synthesis of template-RNA under the conditions of severe restrictions of precursor supply. A parallel situation is, in fact, better seen in phosphate starvation of *S. cerevisiae* in which pulse-labelled RNA of phosphate starved cell has a composition simulating that of DNA. Volkin and Astrachan[36] were the first to demonstrate, in their studies with bacteriophage infection, the incorporation of ^{32}P into an RNA fraction which was distinguishable by its apparent base ratios from the bulk of host bacterial RNA. Spiegelman[26] has shown that during transition of microorganisms from high growth rate to low growth rate medium, there is an instant cessation of net increment in RNA. However, there is an incipient synthesis of RNA having composition similar to the homologous DNA.

Hoagland and coworkers[32] have recently reported data which indicate that 'template RNA' may get associated with a protein of the supernatant fraction resulting in a complex which is very resistant to ribonuclease action. It may well be that the protein is the ribonuclease inhibitor itself. Our data suggest that ribonuclease can act equally efficiently on all the three species of RNA we have isolated, viz. ribosomal RNA, transfer RNA and template-RNA. However, in the native state, ribonuclease action is restricted by cellular location and by ribonuclease inhibitor.

A significant proportion of ribonuclease activity is associated with mitochondrial fraction. Mitochondria have been shown to possess protein-synthesizing apparatus although RNA content of these organelles is very small. We have been exploring further the functional significance of this ribonuclease.

The purpose of presenting these observations is to emphasize that factors controlling RNA turnover may be better understood by an approach which involves growth under conditions of severe limitations on the precursor supply such as protein-fasting or phosphate starvation. Experiments are now underway to examine whether elaboration of ribonuclease under these conditions affects aggregation of ribosomes. It has become increasingly clear in the past year that aggregates of ribosomes (polysomes) are the primary site of protein synthesis in both mammalian[37–39] and bacterial systems[40–42].

SUMMARY

Subcellular distribution of RNases of rat liver and adrenals has been examined. The mitochondrial RNase in both of these tissues exhibits *p*H optima of 5·6 and 7·8. Freezing and thawing of mitochondria or treatment with triton X-100 results in three to four-fold increase in acid RNase of mitochondria whereas mitochondrial alkaline RNase is practically unaffected. Treatment of supernatant fractions with N-ethylmaleimide, an inhibitor of sulfhydryl groups, results in large increases in the supernatant alkaline RNase activity indicating the presence of an inhibitor having free, reactive sulfhydryl groups.

Large amounts of free RNase inhibitor, in excess over the bound alkaline RNase, have been shown to be present in rat adrenals. The inhibitor has been partially purified and shown to be heat labile and non-dialysable.

Levels of alkaline RNase record an increase in protein-fasted rat livers compared to controls, whereas acid RNase level does not show any alteration. The RNase inhibitor registers a fall in activity in protein-depleted animals.

Profile of RNAs as shown by column chromatography on methylated serum albumin-coated kieselguhr columns are altered in protein-fasted rat livers.

Kinetics of incorporation of ^{32}P into different liver RNA fractions shows that an RNA fraction isolated from nuclei and having nucleotide composition simulating that of DNA is more rapidly labelled than transfer RNA and ribosomal RNA. In protein fasting the rapidly labelled RNA is proportionately higher than in normal rats.

The susceptibility of ribosomal RNA, transfer RNA and rapidly labelled RNA towards pancreatic RNase has been examined by following the release of labelled acid-soluble nucleotides from ^{32}P-labelled RNAs. There is no appreciable difference in their susceptibilities.

There is an increase in the activities of acid-phosphatase (phosphomonoesterase) and RNase in *S. cerevisiae* and alkaline phosphatase (phosphomonoesterase) and RNase in *Esch. coli* B_{12}/methionine auxotroph during phosphate starvation. The elaboration of phosphomonoesterase activity can be arrested under conditions which inhibit *de novo* synthesis of proteins whereas elaboration of RNase is not arrested under the same conditions.

Pulse-labelled RNA of *S. cerevisiae* cells grown logarithmically simulates more or less total RNA in nucleotide composition whereas that during phosphate starvation of yeast for 16 hr simulates that of DNA. Phosphate starvation for 16 hr results in net breakdown of RNA although increment in protein continues.

The results are discussed in terms of involvement of RNase in RNA turnover and regulation of protein biosynthesis.

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Role of Hormones in the Regulation of RNA Biosynthesis

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The co-ordination and regulation of metabolic activities in multicellular organisms is mediated by two mechanisms: the central nervous system and the humoral secretions from endocrine glands. In both cases defined chemical agents, in minute concentrations, often of the order of a fraction of a microgram, bring about a chain of characteristic metabolic reactions in target organs. The data to be presented here demonstrate (*a*) that the hormones markedly stimulate the biosynthesis of RNA in target tissues; (*b*) that this stimulation is an early event of hormone action and precedes in time sequence the active phase of protein synthesis; and (*c*) that the secondary cellular events of hormone action are dependent on this initial stimulation of RNA synthesis in the target organs.

The observation that all the three diverse hormone systems studied show an influence on RNA synthesis as an underlying basis of their action, raises the question whether these hormones may not have a direct or indirect role to play in the regulation of RNA synthesis in animal tissues.

The three hormone systems to be discussed are (i) pituitary growth hormone action on liver; (ii) 17β-estradiol action on uterus; and (iii) chorionic gonadotrophin stimulation of the development of immature testis.

Growth hormone is a polypeptide with a molecular weight of 45000 from bovine and 41000 from ovine pituitaries. Both preparations are biologically active in rats. Bovine growth hormone has 396 amino acids. It consists of two polypeptide chains branched perhaps through lysine with 4 S-S bridges between them. The N-terminal amino acids are phenylalanine and alanine and phenylalanine is the C-terminal amino acid. None of these terminal amino acids is essential for the biological activity of the molecule. It is probable that the active core of the molecule is only a small portion of the big chain.

The administration of this hormone to animals leads to a positive nitrogen balance. In liver, the incorporation of (^{32}P)-orthophosphate into ribonucleic acids is greatly increased. This increase is mainly in the nuclear and microsomal fractions and is distinguishable from a generalized increase in all types of RNA occurring in multiplying cells such as the regenerating liver[1]. The nuclear fraction also shows an increased content of RNA. Kinetic studies on the effect of 17β-estradiol on ovariectomized rat uterus show that the rate of incorporation of labelled precursors into uterus RNA increases soon after the administration of a single dose of the hormone and reaches a maximum at 6 hr (Fig. 1). The active phase of protein synthesis reaches a maximum only at 20 hr following the hormone administration (Fig. 2). RNA synthesis thus precedes the protein synthesis and the proliferation of uterine tissue.

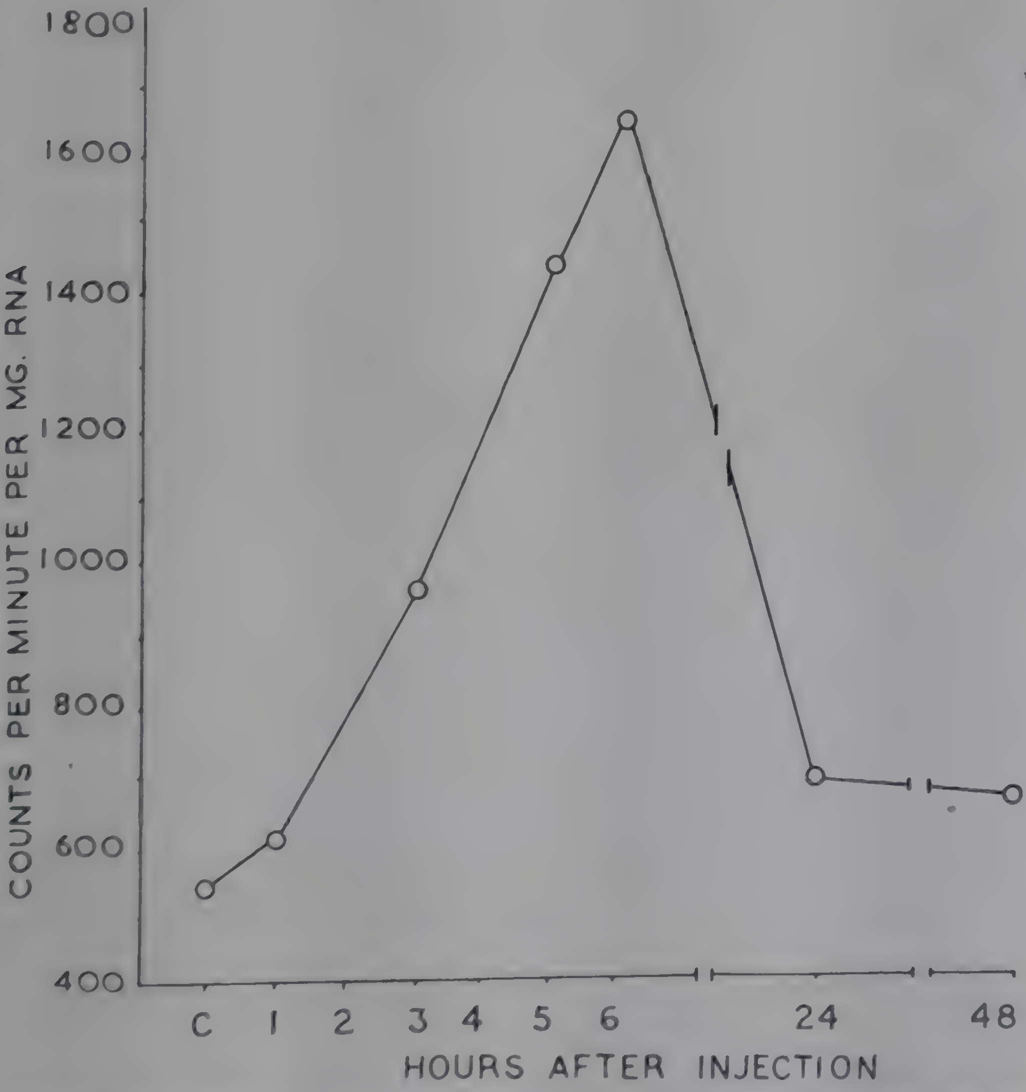

Fig. 1—Rate of incorporation of (^{32}P)-orthophosphate into ribonucleic acids of ovariectomized rat uterus at varying time intervals following the subcutaneous injection of a single dose (5 μg.) of 17β-estradiol: The isotope (100 μC/100 g. body weight) was injected intraperitoneally at the indicated time intervals. Fifteen min. after the isotope injection, the animal was sacrificed and RNA extracted from pooled uteri of 3-6 rats by the method of Davidson and Smellie (*Biochem. J.*, **52** (1952), 399)

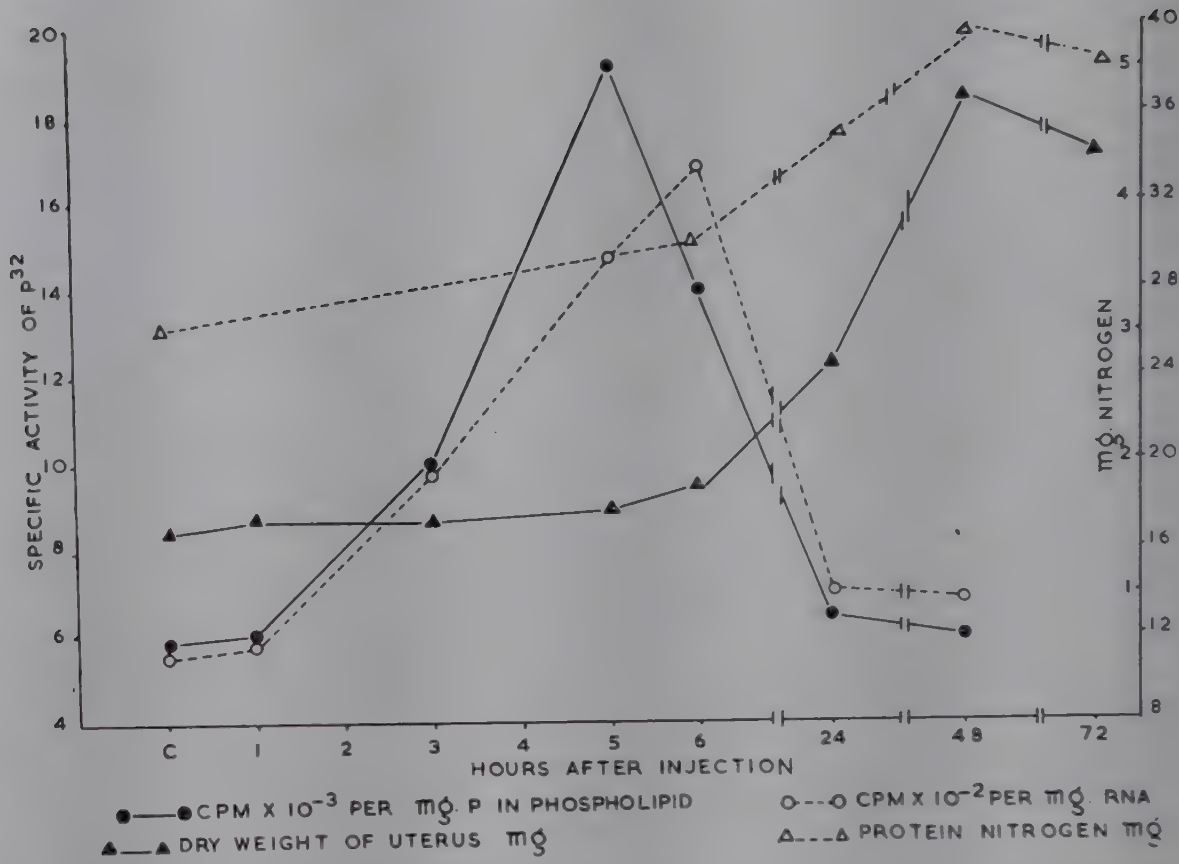

FIG. 2—KINETICS OF BIOCHEMICAL CHANGES IN THE OVARIECTOMIZED RAT UTERUS FOLLOWING ESTRADIOL ADMINISTRATION: 17β-Estradiol (5 μg.) was administered subcutaneously. The uteri were removed at different time intervals for analysis. (^{32}P)-orthophosphate (100 μC/100 g. body weight) was employed as the precursor for assay of the rate of biosynthesis of phospholipids and RNA. Uterus dry mass and protein nitrogen (micro-Kjeldahl's method)were also estimated at different stages of estradiol action

In a further study of the effect of growth hormone on liver, short pulse labelling experiments demonstrated that the stimulation of RNA synthesis was confined to the nuclear fractions in the first instance. When the time of incorporation of the precursor is prolonged, the microsomal fractions of RNA also get highly labelled. Rat liver nuclear RNA has been further subfractionated according to the method described by Sibatani *et al.*[2] The nuclear "ribosomal and transfer RNA" gets relatively less highly labelled than the "nucleolar" fraction obtained by this procedure. A fraction of RNA also gets extracted along with DNA in 1*M* NaCl. The sedimentation characteristics of this fraction on a sucrose density gradient suggest its size to be similar to that of the transfer RNA. This fraction gives an orcinol positive colour reaction. The radioactivity counts are sensitive to alkali and ribonuclease treatment but are resistant to deoxyribonuclease action. The exact nature and function of this fraction are not yet defined. It is, however, not the major peak stimulated by growth hormone.

The "nucleolar" RNA obtained by the procedure of Sibatani *et al.*[2] sediments as a broad peak of polydispersed RNA. The hormone action leads to a very significant rise in the specific activity of some of the peaks associated with this fraction. As mentioned earlier, the stimulation caused by growth hormone originates in the nucleus to begin with, but on prolongation of the time of incorporation of the precursor, the cytoplasmic fractions of RNA in the hormone-treated animals also demonstrate increased specific activity. Analysis of these fractions on a sucrose density gradient from normal and hormone-treated rat livers shows not only a higher specific activity of the precursor in 23(28)S and 16(18)S peaks of ribosomal RNA but also the appearance of very high specific activity in a small peak in between the 4S and 16S peaks. There has been ample evidence both *in vivo* and *in vitro* systems suggesting that the growth hormone administration leads to an increased incorporation of amino acids in rat liver proteins[3]. The hormone, thus, must be elaborating a type of RNA, besides other(s), which is biologically active and leads to an increased synthesis of proteins. Our data show that RNA, the synthesis of which is stimulated by the hormone originates in the nucleus. Some fractions of it get rapidly and highly labelled, and the label passes to the microsomal fractions on prolongation of the pulse time *in vivo*. Actinomycin D inhibits the stimulation caused by growth hormone. These observations suggest that the hormone stimulates the synthesis of messenger type of RNA, besides that of other types.

The sucrose density gradient sedimentation diagrams indicate the polydisperse nature of the RNA stimulated by the hormone. Perry[4] has observed that the nucleolus is the site for the synthesis of rapidly sedimenting heterogeneous RNA, which, on subsequent "chase" incubation, passes to the cytoplasm and is the precursor of the cytoplasmic ribosomal RNA. Our experimental results are consistent with this observation. The growth hormone stimulates the synthesis of the 'nucleolar' RNA fractions, which are the precursors of the microsomal RNA fraction. The hormone seems thus to stimulate both the messenger and ribosomal RNA synthesis in liver (For experimental details of the work on growth hormone described above see Talwar *et al.*[5]).

Dependence of the hormone induced cellular events on RNA synthesis

The results discussed above demonstrate (i) a stimulation of RNA synthesis in the target tissues as an early event of hormone action, and (ii) the nuclear origin of the fractions of RNA, the synthesis of which is stimulated by the hormone. If the ultimate manifestations of hormone action are mediated through this initial stimulation of RNA synthesis, an inhibition of the latter should prevent the sequence of metabolic and cellular events induced by the former. This is indeed the case for estradiol and gonadotrophin action on their target tissues.

Estradiol causes a marked development of the uterine tissue and also specific proliferative changes in the vaginal epithelium. Actinomycin D, an inhibitor of the DNA-dependent RNA synthesis, when applied locally,

TABLE 1—EFFECT OF ACTINOMYCIN ON EVOLUTION OF VAGINAL CYTOLOGY AFTER ADMINISTRATION OF 17β-ESTRADIOL TO OVARIECTOMIZED RATS

Treatment	Concentration of actinomycin	No. of experiments	Changes in vagina	Weight of uterus (mg.)	
				Wet	Dry
None	. .	6	—	84	17
Estradiol (10 μg. S.C.)	. .	6	+	204	49
Estradiol (10 μg. S.C.)	0·5 μg.	3	—	198	48
Estradiol (10 μg. S.C.)	2·0 μg.	3	—	196	47
Estradiol (10 μg. S.C.)	5·0 μg.	6	—	193	47

+ Cornification of the vaginal epithelium.
— No cornified cells in the smear.

TABLE 2—RNA CONTENT OF ACTINOMYCIN-TREATED TESTIS

	Saline	Actinomycin	RNA/DNA
Right	+	—	1·2
Left	—	+	0·6
Right	+	—	1·2
Left	—	+	0·5
Right	+	—	1·4
Left	—	+	0·7

The animals received 60 I.U. HCG (human chorionic gonadotrophin) daily for 6 days. 0·2 μg. of actinomycin in 0·04 ml. saline was injected daily in the left testis, the right testis receiving 0·04 ml. of sterile iso-osmotic saline.

blocks the cornification of the vaginal epithelium (Table 1). This effect is not due to the antibiotic properties of the compound, as streptomycin and penicillin in much higher concentrations are ineffective as blocking agents[6]. Besides, the uterus responds normally to the injected hormone while vaginal epithelium development is blocked locally.

Similar results are observed in another hormone system. Chorionic gonadotrophin administration to immature male rats leads to the development and growth of the testis. If actinomycin-D in minute concentrations is administered to one of the testes, while the other testis receives an equal volume of sterile iso-osmotic saline, a significant inhibition in the growth of the former is observed as compared to the latter in response to the gonadotrophin injected. The doses of actinomycin sufficient to cause this effect are low and do not exert any toxic effect on the tissue, as evidenced by histological examination and recovery experiments. The actinomycin-blocked testis is able to respond to the gonadotrophin influence normally when actinomycin administration is stopped. Table 2 shows the RNA levels per unit DNA in the saline and actinomycin-treated testes. An inhibition of the RNA synthesis thus masks the effects of hormone action.

How does the hormone stimulate RNA synthesis ?

The experimental data presented permit the conclusion that the hormones studied stimulate the biosynthesis of two or more types of ribonucleic acids, which require DNA as a primer for their synthesis. Does the hormone act at the level of RNA polymerase ? To investigate this possibility, the activity of this enzyme was assayed in preparations from normal and growth hormone-treated rat livers. No significant difference was, however, discernible in the two cases (S. Bourgeois-Cohn and G. P. Talwar, unpublished data). Similar observations have also been made by J. Hurwitz (personal communication).

Recently Tissieres *et al.*[7] have reported a marked inhibition of RNA polymerase activity by various kinds of RNA. The inhibition produced by soluble RNA (s-RNA) was considerably less when this RNA was charged with amino acids. The amino acid pool in the cell could thus influence the RNA polymerase activity *in situ* through the concentration of free s-RNA.

It is known that some hormones, such as insulin, cause a change in the permeability of the tissues to specific metabolites. When estradiol is injected into ovariectomized rats, a large imbibition of water is noticed in the uterus in the early phases. This could be accompanied by permeability changes to other metabolites as well. Knobil has also reported an increased uptake of amino acids in rat diaphragm treated by growth hormone. We have investigated the total pool of free amino acids in livers of normal and growth hormone-treated rats (Table 3) and in the ovariectomized rat uterus, before and 6 hr after estradiol administration (Table 4). The pool size in the hormone-treated tissues is not increased in either case. It is, therefore, unlikely that the control mechanism be operating through the amino acid-charged moiety of transfer RNA in the hormone-primed tissues.

Another property of RNA polymerase prepared from bacterial or mammalian cells is the requirement for all four nucleoside triphosphates. The limiting concentration of any one of the four nucleotides could limit the rate of RNA synthesis. The work of Mueller and collaborators has shown that the pool size of uridine nucleotides increases about 10 to 12-fold in the uterus after estradiol treatment. Some preliminary results on the aspartate transcarbamylase activity in the normal and growth hormone-treated rat liver demonstrate an increase in the activity of this enzyme in the hormone-treated tissues (Table 5). Further work is in progress to see whether this activation is specific or a part of the generalized increase in several cytoplasmic enzyme activities.

TABLE 3—FREE AMINO ACID POOL OF RAT LIVER

[Ovine growth hormone (3 mg./100 g. body weight) in sterile iso-osmotic saline was administered intraperitoneally. The animals were sacrificed 5 hr after the hormone injection. TCA-soluble material was extracted from the livers. TCA was removed from the extract by ether. The total amino acid pool was estimated in the extract by the method of Moore and Stein (Figures in parenthesis denote the number of experiments)]

	μmoles leucine equivalents/g. of fresh weight
Control (plateau rats)	22·6 (2)
Plateau rats + growth hormone	22·05 (2)
Control (3 weeks rats)	20·6 (3)
3 weeks rats + growth hormone	18·7 (3)

TABLE 4—TOTAL FREE AMINO ACID POOL OF OVARIECTOMIZED RAT UTERUS

[Estradiol (10 μg.) was injected in the ovariectomized rats subcutaneously and the uteri were removed 6 hr after hormone administration. The free amino acid pool was extracted and estimated as described in the legend of Table 3]

	μmoles leucine equivalents	
	per g. tissue protein	per g. fresh weight
No treatment	430*	27·7
Estradiol	426**	25·1

* Average of 16 rats
** Average of 10 rats.

TABLE 5—ASPARTATE TRANSCARBAMYLASE ACTIVITY IN LIVERS OF NORMAL AND GROWTH HORMONE-TREATED RATS

[Ovine growth (hormone 3 mg./100 g. body weight) was injected intraperitoneally to female plateaued rats. The livers were removed 6 hr after hormone administration. The tissues were homogenized in 0·04M KCl–0·04M $NaHCO_3$. The supernatant obtained after centrifuging at 15,000 g for 20 min. was passed through Sephadex G-50, equilibrated with phosphate buffer ($2 \times 10^{-2} M$, pH 7) containing $2 \times 10^{-3} M$ of mercaptoethanol and $2 \times 10^{-4} M$ EDTA. The enzyme activity was assayed by the method of Gerhart and Pardee[8]]

	mμmoles carbamyl aspartate/mg. protein/30 min.		
	Exp. I	Exp. II	Exp. III
Normal	60·6	61·2	36·4
G. H.	82·2	89·4	48·2
% Increase	35·6	46·0	32·4

Acknowledgement

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Studies on the Amino Acyl Adenosine Endgroup of Amino Acyl s-RNA*

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In an intermediate step of protein biosynthesis the amino acids are bound to s-RNA[1]; they are linked by an ester bond to the terminal adenosine[2-4]. Structural studies on the amino acyl adenosine endgroup of amino acyl s-RNA will be described showing the 3′-hydroxyl group of this adenosine to be the point of attachment of the amino acids. In the second part of this paper, experiments with model compounds are summarized, which may help to explain the high reactivity of the ester bond in amino acyl s-RNA.

Localization of the ester bond between amino acids and s-RNA

The problem whether amino acids are attached to the 2′- or to the 3′-position of the terminal adenosine of s-RNA, was discussed by Zamecnik[5] as one of the "unsettled questions in protein synthesis". On the basis of reactivity it was argued that the 2′-hydroxyl group of the terminal adenosine of s-RNA is the more likely[6] or at least the initial[5] point of attachment of the amino acids. On the other hand, the effect of puromycin[7] on protein synthesis and particularly the experiments with puromycin analogues[8] could be interpreted in favour of a 3′-linkage. Theoretical considerations on the specificity in protein synthesis and on the stereochemistry of the ester bond in amino acyl s-RNA also pointed towards a 3′-linkage[9]. Chemical reactions with amino acyl adenosine isolated from amino acyl s-RNA with basic ion exchangers always yielded 1:1 to 1:2 mixtures of 2′- and 3′-derivatives[10-12]. The migration of the acyl group between the neighbouring hydroxyl groups is possible or even likely under most of the conditions used in the isolation of the compound and in the chemical reaction

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* Abbreviations: s-RNA = soluble ribonucleic acid; n.m.r. = nuclear magnetic resonance; AMP, UMP, GMP, CMP = mononucleotides.

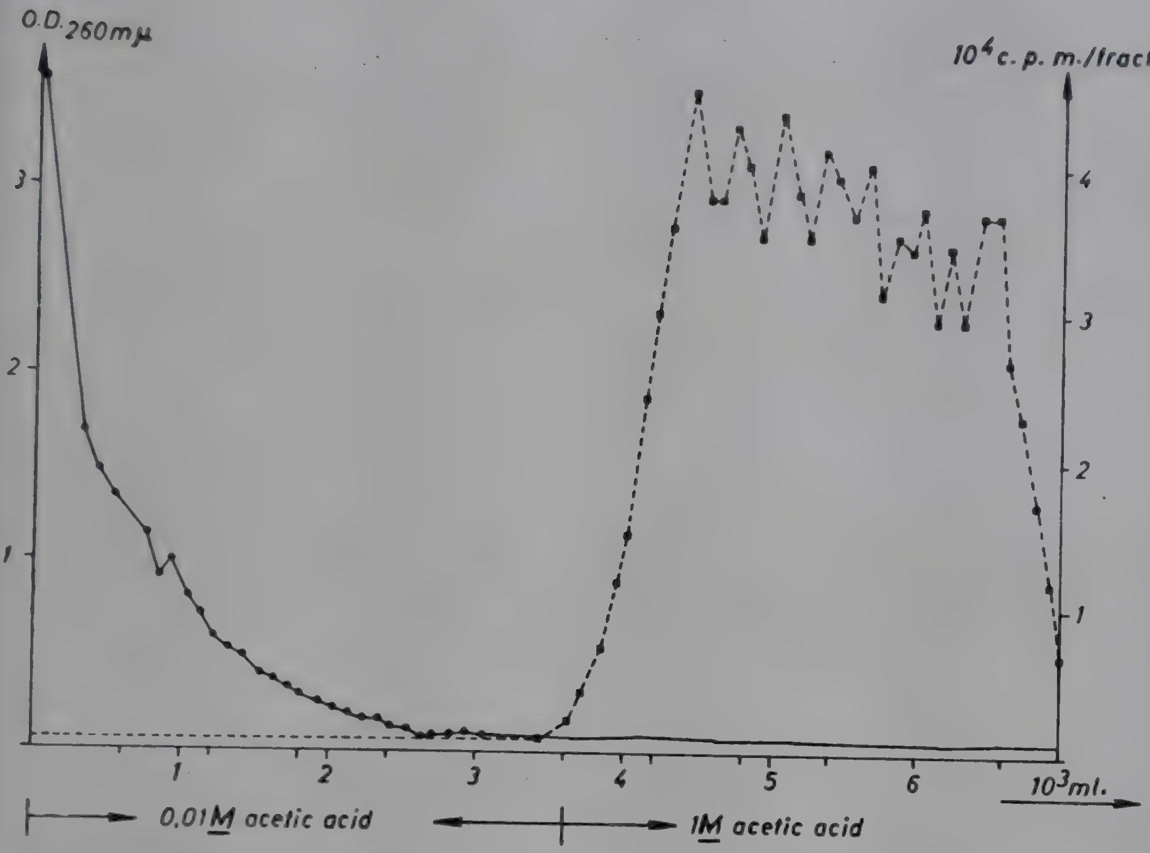

Fig. 1—Isolation of 12 μmoles amino acyl adenosine from 9 g. amino acyl s-RNA by chromatography on an Amberlite IR-C 50 column (1·5×40 cm.)

sequences. The possibility of acyl migration has been considered by everybody working with this class of compounds and has been particularly emphasized by some authors[5,10-13].

Since acyl migration appeared to be more likely under alkaline and strongly acidic conditions than at neutral or slightly acidic *p*H, we recently isolated amino acyl adenosine from amino acyl s-RNA with the help of the weakly acidic ion exchange resin Amberlite IR-C 50 (Fig. 1). This amino acyl adenosine was compared with valyl adenosine, which was prepared[14] by a method involving chemical acylation under vigorous conditions and in the last step also a chromatography on Amberlite IR-C 50. Both compounds were investigated by n.m.r. spectroscopy[15] and subjected to chemical reactions[16].

N.m.r. spectroscopy seemed to be a promising method for determining the position of the ester bond, since the resonance signals of the protons in the ribose part of adenosine have been identified[17]. The esterification of a hydroxyl group then shifts to lower fields, those resonance signals which belong to neighbouring C-H bonds. The resonance signals of adenosine, 2′/3′-cyclic AMP, 2′-AMP and 3′-AMP were determined; on this basis the resonance signals of synthetic valyl adenosine and amino acyl adenosine from amino acyl s-RNA could be identified. Synthetic valyl adenosine was found to be an approximately 1 : 2 mixture of the 2′- and the 3′-ester. On the other band, amino acyl adenosine from amino acyl s-RNA showed only bands characteristic the 3′-ester and only 5% of the 2′-ester could be detected. The experimental data and the spectra have been published[15] and will not be repeated here.

When amino acyl adenosine was isolated from amino acyl s-RNA with a basic ion exchanger and then was carried through the series of reactions shown in Fig. 2, a 1:1 mixture of 2′- and 3′-derivatives was obtained[12]. When on the other hand Amberlite IR-C 50 was used for isolation and the conditions of the subsequent reactions (Fig. 2) were slightly modified, 93% of a derivative arising from 3′-amino acyl adenosine was found[16]. Synthetic valyl adenosine under the same conditions yielded a 30:70 mixture of 2′- and 3′-derivatives. For both the natural and the synthetic compounds, the agreement between the n.m.r. data and the results of the chemical reactions is good. The amino acyl s-RNA in the n.m.r. experiments was charged with 7 and the amino acyl s-RNA for the chemical studies with 14 different amino acids. It is important to note that the amino acyl s-RNA investigated here is biologically active in the ribosomal system and also in the enzymatic deacylation, i.e. in the back reaction of the initial acylation step.

When synthetic valyl adenosine is kept in pentadeutero pyridine (for 58 hr) or in hexadeutero dimethyl sulfoxide, it remains a 1 : 2 mixture of 2′- and 3′-esters. So this compound seems to be an equilibrium mixture of the isomers as expected from its synthesis. When amino acyl adenosine from amino acyl s-RNA is kept in hexadeutero dimethyl sulfoxide (containing some D_2O) for 2-3 days, the bands belonging to the 2′-ester appear in the n.m.r. spectrum. It was, therefore, concluded[15,16] that the 3′-amino acyl adenosine itself is not the product of an acyl migration, but the primary product derived from a 3′-amino acyl s-RNA. In some model compounds

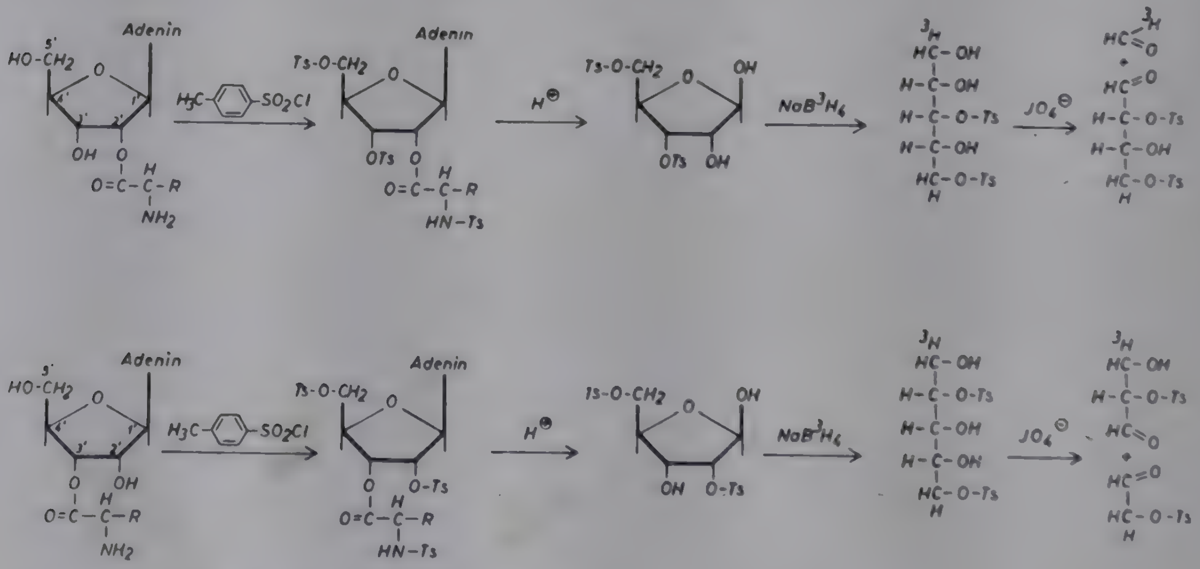

FIG. 2—A SERIES OF REACTIONS FOR THE LOCALIZATION OF THE ESTER BOND IN AMINO ACYL ADENOSINE[12,16] (Ts=*p*-toluene sulfonyl)

(loc. cit.[11] and C. S. McLaughlin, personal communication), however, acyl migration is a fast reaction in aqueous solution under physiological conditions. It is not certain whether this applies also to the compounds investigated here. If it does, 93-95% of the 3′-ester in amino acyl adenosine from amino acyl s-RNA would be the concentration in an equilibrium mixture. In this case one would have to assume that for a few amino acid esters, the 3′-ester content is lower (70% for the valine ester) but that for the majority of them the linkage is to the 3′-hydroxyl group of adenosine in more than 93-95%. Therefore, our conclusion that the amino acids are linked to the 3′-position of the terminal adenosine of s-RNA under physiological conditions, holds at least for the majority of the amino acids, also in the case of a fast acyl migration previous to dissolving in an organic solvent. It will be of interest to determine the equilibrium concentration of 2′- and 3′-isomers in different amino acid esters of adenosine.

Todd and coworkers[18] found that acetylation or benzoylation of nucleosides always forms 3′, 5′-diacyl derivatives; 2′, 5′-diacyl compounds could not be isolated. Evidence from tosylation, on the other hand, showed that the 2′-position is more reactive than the 3′-position. Since the tosyl group does not migrate, it was concluded that the initial site of reaction in all cases is the 2′-hydroxyl group[18]. On the basis of this work the above mentioned hypothesis[5], that is, in protein synthesis the 2′-hydroxyl group of the terminal adenosine of s-RNA is the initial site of acylation by the activated amino acids, was stated. Postulating this, one would have to assume a quantitative or nearly quantitative migration of most amino acids from the 2′- to the 3′-position, while the amino acyl s-RNA is still bound to the enzyme or when it is released from the enzyme. This possibility cannot be excluded. Direct acylation of the 3′-hydroxyl group, however, is also possible, since the activated amino acids react from an enzyme-bound form and enzymatic specificity may be more important than chemical reactivity. In any case, the experimental fact is that the majority of the amino acids are found to be linked to the 3′-position when the amino acyl s-RNA is released from the enzyme and is ready for protein synthesis.

Reactivity of the ester bond between amino acids and s-RNA

The free energy of hydrolysis of the ester bond in amino acyl s-RNA is similar to that of the cleavage of a pyrophosphate bond in adenosine triphosphate[3,19]. This "energy rich" character of the ester bond can be understood on the basis of comparative thermodynamic measurements[20] and general stereochemical considerations[9]. The ester bond also exhibits a rather high reactivity; leucyl s-RNA is hydrolyzed 30 times faster than leucine ethyl ester[21] and in the reaction of the two compounds with hydroxylamine the difference in reactivity is so large[2] that the rates cannot be compared accurately under the same conditions. It seems of interest to summarize some work on the structural reasons for the high reactivity of

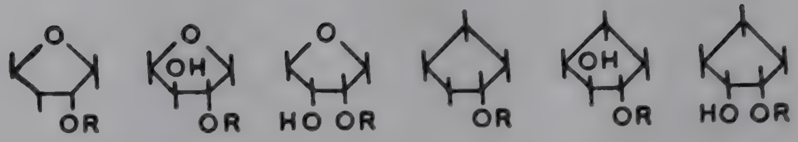

FIG. 3—MODEL COMPOUNDS[27,28] (R=acetyl-, glycyl-, valyl-)

the ester bond in amino acyl s-RNA. Most of the work was done by comparing this reactivity with the reactivities of model compounds.

An effect of a free hydroxyl group vicinal to the esterified hydroxyl group on the reactivity of the ester bond had to be expected from textbook[22] knowledge. The assistance in the alkaline hydrolysis by a neighbouring hydroxyl group ("Henbest[23]-Kupchan[24]-effect")[25,26] was attributed partly to an intramolecular hydrogen bond[23]. To study the effect on compounds structurally related to nucleoside esters, some time ago the monoesters of acetic acid, glycine and valine with cyclic alcohols (cyclopentanol, 3-hydroxytetrahydrofuran) and diols (*trans*- and *cis*-3,4-dihydroxytetrahydrofuran, *trans*- and *cis*-1, 2-dihydroxycyclopentane) (Fig. 3) were synthesized[27]. The rate constants of the hydrolysis and of the reaction with hydroxylamine were determined. Numerous data[27] show the significant influence of the neighbouring hydroxyl group on the reactivity of the ester bond, an influence, which is greater in the *cis*- than in the *trans*-compounds (Table 6 in loc. cit.[27]). A difference between the leucine esters of *trans*- and *cis*-dihydroxycyclopentanes in the rate of hydroxamic acid formation was also observed[6].

An intramolecular hydrogen bond was found to exist in the acetic acid ester of *trans*-dihydroxycyclopentane but not in the corresponding *cis*-ester and not in the esters of the dihydroxytetrahydrofurans. The spectra[28] have been discussed before (footnote on pp. 1835 in ref. 27); they are given here for full documentation (Fig. 4). Also in the corresponding glycine ester series only the ester of the *trans*-dihydroxycyclopentane seems to have an intramolecular hydrogen bond, as was made probable[28] by comparing relative rates of hydrolysis at pH 9·8 and 11·5. The *trans*-ester becomes less reactive (decreasing $k_{rel.}$) as compared with the *cis*-ester ($k_{rel.} = 1{\cdot}0$), when the hydrogen bridge is weakened at higher pH (cf. Table 1).

From the studies with model compounds it can be inferred with some certainty that the high reactivity of the 3′-amino acyl adenosine endgroup of amino acyl s-RNA is at least partly due to the adjacent 2′-hydroxyl group. Certainly inductive and steric effects and possibly hydrogen bridges are involved here.

A second factor influencing the reactivity of the ester bond is the oxygen in the 5-membered ring of the alcohol moiety (Table 5 in ref. 27). The glycine ester of *cis*-3, 4-dihydroxytetrahydrofuran for instance is hydrolysed 6·7 times faster and reacts 39 times faster with hydroxylamine than the corresponding dihydroxy cyclopentane ester[27]. An inductive effect of the ring oxygen may be one reason; another reason could lie in finer

TABLE 1—Dependence of Relative Rates of Hydrolysis on *p*H

[Indications were obtained from infrared spectra that hydrogen bonding exists in nucleoside esters[29]. Recently intensive studies on intramolecular hydrogen bonds in diol acetates were reported[26]. The reactivities of amino acid esters of ethylene glycol and glycol phosphate have been studied[30]. The important role of neighbouring hydroxyl group assistance was stressed by comparing rates of hydrolysis of ribo- and deoxyribonucleoside esters[31]]

		Hydrolysis	
Glycine esters of		at *p*H 9·8 $K_{rel.}$	at *p*H 11·5 $K_{rel.}$
Dihydroxycyclopentane	*trans*	0·83	0·56
	cis	1·0	1·0
Dihydroxytetrahydrofuran	*trans*	0·34	0·37
	cis	1·0	1·0

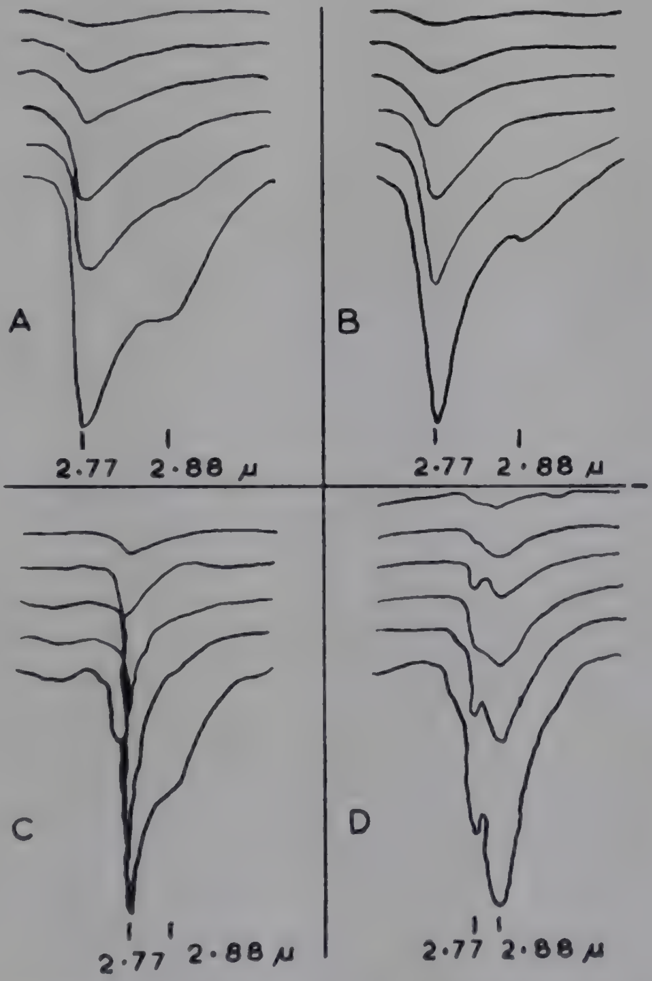

Fig. 4—Bands corresponding to OH-stretching vibrations of monoesters of acetic acid with *trans*- and *cis*- 3, 4-dihydroxy tetrahydrofuranes (A and B) and *cis*- and *trans*-1, 2-dihydroxycyclopentanes (C and D)[27,28]: The infrared spectra were taken in CCl_4 at different concentrations with a Perkin-Elmer Model 21 with CaF_2-optics (For measurements and discussions thanks are due to Miss J. Kohler and Dr E. Bickert respectively)

details of conformation in cyclopentane and tetrahydrofuran derivatives, as was suggested by Dr A. M. Michelson (personal communication). Possibly, there is some connection between this effect and the above mentioned lack of hydrogen bridging in the tetrahydrofuran derivatives.

The effects of the base on C-1 and/or the hydroxymethyl group on C-4 of the ribose on the reactivity of a nucleoside ester cannot be overlooked; valyl adenosine is hydrolysed 5 times faster and reacts 8·7 times faster with hydroxylamine than the valine ester of *cis*-3, 4-dihydroxy tetrahydrofuran[27]. Again inductive and conformational effects may play a role here. It does not seem to matter much which base is attached to the ribose and whether or not the nucleoside carries a phosphate group or a whole RNA chain. The rate constants for hydrolysis and hydroxylamine reaction of the valine esters of s-RNA, 5′-AMP and the four ribonucleosides show some interesting differences (Table 1 in ref. 14), but on the whole the differences are small (±20%) compared with the above-mentioned effects. Also for leucyl-AMP, -UMP and -GMP very little variations in the rate of the hydroxylamine reaction have been found[6]. These results do not exclude the possibility that the adenine moiety of the terminal nucleoside in s-RNA plays a particular role in the enzymatic esterification reaction[5], but this may be related to the specificity of the activating enzyme rather than to a special influence of the adenine on the ribose. It should be emphasized that the studies[6,14,37] with synthetic amino acyl nucleosides and nucleotides were done with mixtures of the 2- and the 3-isomers. In a more detailed study this point would have to be taken into consideration.

For the sake of completeness the nature of the acyl group should be mentioned as one of the major factors which influence the reactivity of an ester bond (Table 7 in ref. 27). In general, amino acid esters are more reactive than the corresponding carboxylic acid esters. Within the class of amino acid esters there are large differences in reactivity depending on the nature of the amino acid. Glycine esters of *trans*- and *cis*-dihydroxy tetrahydrofuran are hydrolysed 8 times faster and react with hydroxylamine 13-16 times faster than the corresponding valine ester. Similar differences are found between different amino acid s-RNA esters.

The model compounds discussed above cover with respect to reactivity, the whole range from the "normal" amino acid alkyl esters to amino acyl s-RNA. In conclusion it may be said that the high reactivity of the ester bond between amino acids and s-RNA is mainly (but perhaps not exclusively) due to 4 factors: the neighbouring *cis*-hydroxyl group, the ring oxygen in the ribose, the base on C-1 of the ribose, and the nature of the particular amino acid.

SUMMARY

According to n.m.r. spectra and chemical degradation studies the amino

acids are esterified to the 3′-hydroxyl group of the terminal adenosine of s-RNA (93-95% 3′-ester). The high reactivity of this ester bond is largely due to the neighbouring *cis*-hydroxyl group, the ring oxygen in the ribose and the base on C-1 of the ribose. The work of the authors in this field is summarized and discussed in relation to recent publications by other authors.

Acknowledgement

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Preparation of Transfer RNA Polypeptides and Some of Their Properties

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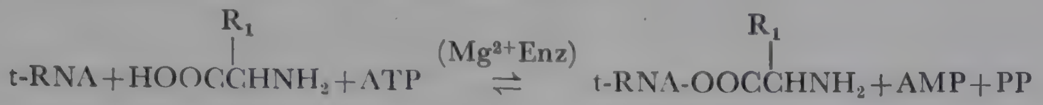
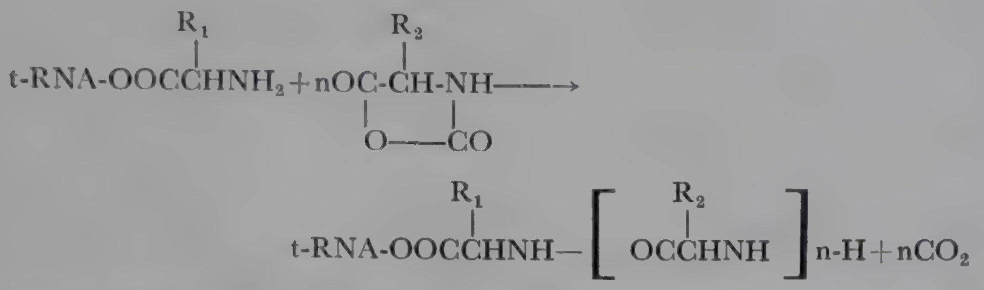
It is now generally believed that the formation of aminoacyl transfer RNA is one of the intermediate steps of protein synthesis. Aminoacyl RNA molecules transfer their amino acid moieties into growing polypeptide chains. Further more, the amino acid sequence is probably determined through the formation of specific hydrogen bonds between a specific nucleotide sequence in a messenger RNA molecule and a complementary sequence in the t-RNA molecule. It is also known that for any given amino acid there is at least one molecular type of t-RNA specific to it. In some instances it has been shown that several molecular species of t-RNA specific for single AA exist. Thus, in each type of t-RNA there is a specific region recognized by a particular aminoacyl RNA synthetase for the purpose of linking the appropriate amino acid to RNA and there is also a nucleotide sequence for reading the genetic code. Therefore, it becomes of interest to try and isolate specific amino acid acceptor RNA chains.

Several attempts to isolate amino acid specific t-RNA have been reported in the literature[1-3]. Partial purification was achieved by chromatography on anion exchangers[4,5], electrophoresis[6] and by partition chromatography[7] on Sephadex G-25. Holly and coworkers[8,9] using the countercurrent distribution technique, have obtained almost complete separation of tyrosyl and alanyl-RNA chains[10]. Another technique has been to employ periodate oxidation of all but one type of RNA chain followed by purification of the unoxidized aminoacyl RNA via fractional precipitation[11,12] or chromatography on aminoethylcellulose columns[13].

We have attempted for some time[14,15] to employ N-carboxyanhydrides for separating single species of t-RNA. A single amino acid was bound enzymatically to t-RNA (Eq. 1) and the primary aliphatic amine of the resulting aminoacyl RNA (RNA-AA) was used to initiate the polymerization of a suitable N-carboxy α-amino acid anhydride leading to the formation of the polypeptidyl t-RNA derivative (Eq. 2).



$$\text{t-RNA}+\text{HOOC}\overset{R_1}{\text{C}}\text{HNH}_2+\text{ATP} \overset{(\text{Mg}^{2+}\text{Enz})}{\rightleftharpoons} \text{t-RNA-OOC}\overset{R_1}{\text{C}}\text{HNH}_2+\text{AMP}+\text{PP} \quad ..(1)$$



$$\text{t-RNA-OOC}\overset{R_1}{\text{C}}\text{HNH}_2+\text{n}\underset{\text{O}\text{——}\text{CO}}{\text{OC-}\overset{R_2}{\text{C}}\text{H-NH}} \longrightarrow$$

$$\text{t-RNA-OOC}\overset{R_1}{\text{C}}\text{HNH—}\left[\text{OC}\overset{R_2}{\text{C}}\text{HNH}\right]\text{n-H}+\text{nCO}_2 \quad ..(2)$$

Early attempts in our laboratory[16] and by Zachau[17] to separate the RNA-AA polypeptide from the rest of the unreacted RNA chains yielded only a limited degree of purification. This was due to the presence of some tightly bound proteins in the t-RNA preparation which acted as initiators of the anhydride polymerization. Using t-RNA preparations with low protein contents and preparing water insoluble t-RNA-AA polypeptide derivatives from them a somewhat better purification was achieved[14,15]. More recently similar method has also been used by Mehler and Bank[18].

Several N-carboxyanhydrides can be used in this fractionation procedure of amino acid specific t-RNA. Thus, when the polymerization of the N-carboxyanhydrides of β-benzyl-L-aspartate, ε-carboxy-L-lysine, L-phenylalanine or L-leucine is initiated in aqueous medium by t-RNA previously charged with a given single-labelled amino acid, the bulk of the radioactivity precipitates out from the reaction mixture, in the form we think of t-RNA-AA polypeptide. N-carboxyanhydrides of β-benzyl-L-aspartate were found to give the best results. The use of this method rests on several factors and these will be dealt with in detail below.

t-RNA preparation. The t-RNA used was isolated from whole brewer's yeast cells or from *Esch. coli* by the phenol procedure of Monier *et al.*[19] and Zubay[13]. Bound amino acids were removed by incubating the t-RNA in 0·2 *M* Tris buffer (*p*H 8·8) for 5 hr at 37°C. The RNA was then freed from ribosomal RNA, small oligonucleotides, polysaccharides and polyphosphates by DEAE fractionation. Such isolated RNA preparations still contained about 2·5% protein-like (peptide) material as measured by the phenol method[16]. This "peptide" probably also acts as an initiator of the anhydride polymerization. Thus when *Esch. coli* ^{32}P-t-RNA was used, about 1·2% of the label precipitated after reaction with N-carboxy-β-benzyl-L-aspartate anhydride. Since the amount of the different types of amino acid specific RNAs ranges between 0·5 and 5%, the presence of bound peptide in the RNA preparation will produce a considerable contamination of non-specific RNA in the final precipitate and will reduce significantly the purity of the RNA-AA polypeptide obtained. The nature of the binding of this "peptide" to t-RNA is not clear. The possibility that the proteinaceous contaminant in t-RNA preparations is in fact a naturally occurring t-RNA polypeptide is not necessarily surprising in view of the current concept of polypeptide synthesis, and might explain the fact that "peptide" contamination in ribosomal RNA is considerably lower (about 0·5%)[20]. The protein content was reduced to a value of about 1·5 to 2·0% by passing the t-RNA through an IRC-50 column in 0·1 *M* Sodium acetate buffer, *p*H 3·5[21]. The true amount of protein might be lower since the values were not corrected for background reaction due to nucleotides. The possible interference by residual phenol must also be considered. Using, again, ^{32}P-t-RNA, the amount of label precipitated by the anhydride was found to be only 0·7%. While the IRC-50 treatment of the RNA somewhat reduced the reaction between the N-carboxyanhydride and uncharged t-RNA, more work is obviously needed.

The presence of traces of bound nucleases in RNA preparations has been mentioned previously[20,22,23]. It is, therefore, important to establish that the t-RNA preparations are free of nuclease contamination which survived the treatment with phenol. As a routine test the t-RNA was dissolved in 0·2 *M* NaCl, heated for 30 min. at 60°C. and amino acid acceptor ability assayed (Holley, private communication). No decrease in activity was found, indicating that our preparations are practically free of nuclease contamination.

Source of enzyme. For the esterification of yeast t-RNA with amino acid we used a rat liver enzyme preparation ("streptomycin supernatant fraction")[24]. The free amino acids present in the enzyme mixture were removed immediately before use by Sephadex G-50 gel filtration. We were unable to use yeast cell extracts as an enzyme source since their amino-acyl RNA synthetases were rather unstable and, therefore, rat liver was used as the enzyme source. For some amino acids the use of a rat liver activating enzyme might prove ineffective with yeast t-RNA[25]. The possibility also exists that during incubation with the crude rat liver enzyme some amino acids might be released (e.g. through autolysis) and, if so, would become linked to the t-RNA preparation added. Finally the rat liver enzyme contains traces of nucleases which decrease the activity of the t-RNA by about 10%. To reduce these difficulties we have recently used *Esch. coli* RNA and highly purified *Esch. coli* aminoacyl RNA synthetase[26]. The amino acids used are of the DL-type since some L-amino acid preparations are contaminated with other amino acids.

Stability of aminoacyl RNA. In order to obtain good yields of RNA-AA-polypeptide the aminoacyl RNA derivatives should be stable at the *p*H values used for the polymerization reaction (about *p*H 7·5). It has been shown that the rates of hydrolysis of the different aminoacyl RNAs vary considerably[27] (Muench and Berg, private communication). Thus, glycyl, arginyl, glutamyl and aspargyl RNA are considerably less stable to hydrolysis than seryl or valyl RNA, at pH 7·5 and 37°C. The yield of the various aminoacyl-RNA polypeptides will, therefore, vary with the amino acid employed. In order to minimize hydrolysis, the reaction with carboxyanhydrides was carried out at pH 7·5 and 0°C.

Preparation of t-RNA-AA-polypeptides. The experimental procedure used is illustrated by the synthesis of t-RNA-^{14}C-serine-poly-β-benzyl-L-aspartate. In this case β-benzyl-N-carboxy-L-aspartate anhydride[28] was used as a monomer and the polymerization initiated with t-RNA-^{14}C-serine. Labelled serine was bound enzymatically to yeast t-RNA and the isolated ^{14}C-serine-t-RNA was dissolved in 1·0 ml. of 0·02M NaCl. The aqueous solution was mixed with 25 mg. of N-carboxy-β-benzyl-L-aspartate anhydride dissolved in 0·6 ml. dioxane. The reaction mixture was incubated at 0°C. for 2 hr and the precipitate formed was centrifuged and washed with water to remove adsorbed RNA. The amount of RNA in the precipitate was determined from its phosphorus content while the amount of serine was estimated from the difference between the radioactivity of the original reaction mixture and that of the supernatant. This precipitate was found to contain 20 mμmoles serine per mg. RNA as will be shown later. An assay for amino acid acceptance of the t-RNA left in the supernatant showed that the binding for serine decreased by an amount equal to the ^{14}C-serine in the precipitate. On the other hand, no significant change could be detected in the binding capacity of alanine, leucine or threonine.

The t-RNA-ser-poly-β-benzyl aspartate, formed in the polymerization mixture, was isolated from the precipitate containing poly-β-benzyl-L-aspartate and small amounts of ^{14}C-ser-poly-β-benzyl aspartate, with the aid of a DEAE-cellulose column (Fig. 1). The precipitate was dissolved in dimethylformamide and passed through a DEAE-cellulose column saturated with dimethylformamide. The poly-β-benzyl-L-aspartate and ^{14}C-ser-poly-β-benzyl aspartate were removed by washing the column with dimethylformamide and the t-RNA-ser-polypeptide then eluted with aqueous 1 M NaCl. The aqueous eluate was dialyzed against water and assayed for radioactivity and RNA. It was found to contain 18 mμmoles serine per mg. RNA. It should be noted that t-RNA-ser-polypeptide emerging from the column is soluble in water. The fact that it precipitated out of the initial polymerization mixture may be due to the presence of dioxane in the reaction mixture or co-precipitation with the large amount of poly-β-benzyl-L-aspartate which was also formed during the reaction.

Control experiments revealed that β-benzyl-N-carboxy-L-aspartate when reacted with t-RNA freed of bound amino acids did not decrease the amino acid binding capacity of the RNA for serine, alanine, leucine and threonine indicating that t-RNA is not affected significantly by the above treatment.

Separation of seryl, alanyl and threonyl RNA. The efficiency of the procedure given for the separation of three different aminoacyl

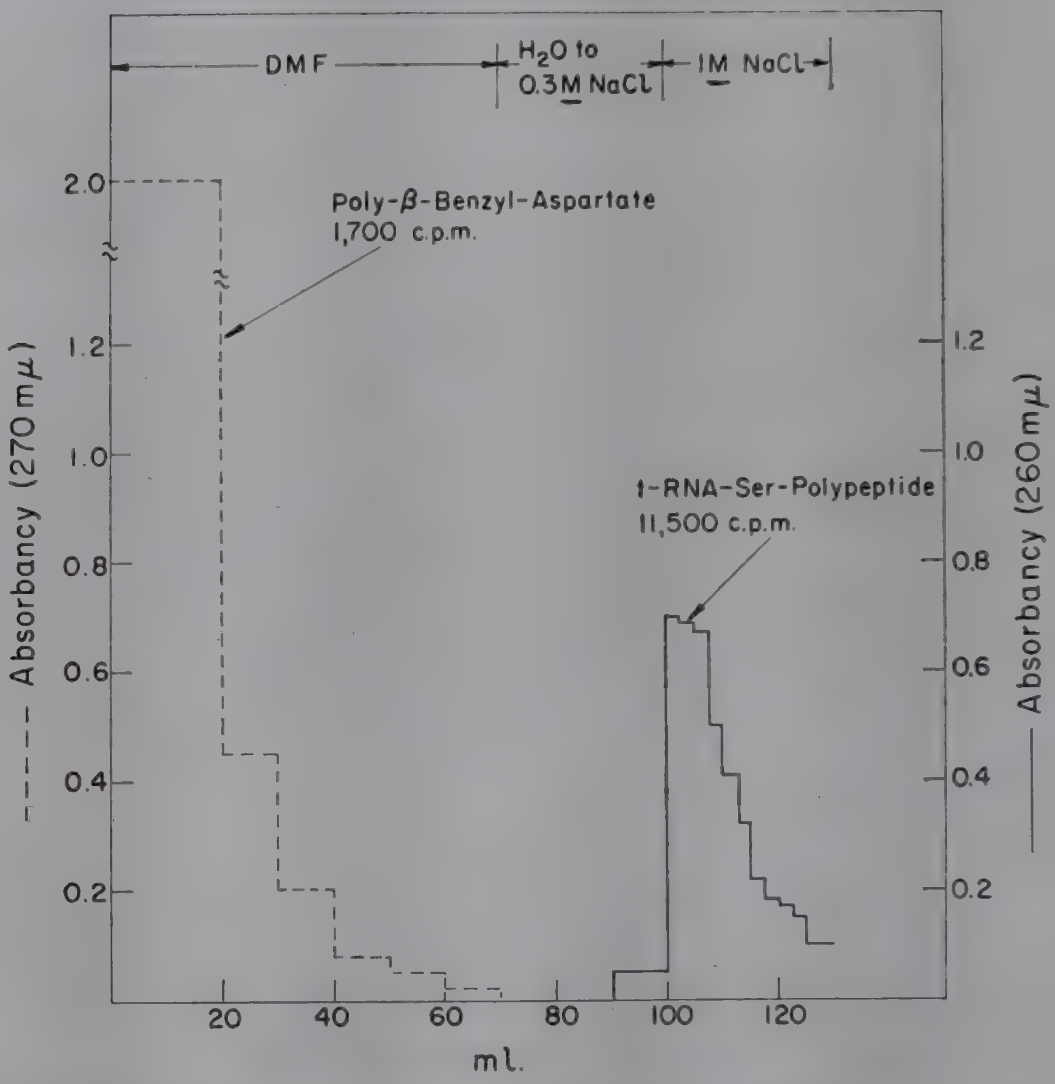


FIG. 1—CHROMATOGRAPHIC PURIFICATION OF t-RNA-SERINE-POLY-β-BENZYL-L-ASPARTATE

t-RNAs from the original yeast t-RNA mixture is summarized in Table 1. The precipitates formed with t-RNA-serine-^{14}C, t-RNA-alanine-^{14}C, and t-RNA-threonine-^{14}C contained 50-70% of the initial counts and 4-6% of the initial t-RNA. The loss in acceptance for the specific amino acids tested in the t-RNA left in the supernatant (40-60%) was somewhat lower than that to be expected from the amount of labelled amino acid precipitated (compare the data of column 3 with the corresponding data of columns 5-7). This may be due to the partial hydrolysis of the aminoacyl t-RNAs under the conditions employed, and the initiation of the polymerization of the N-carboxyamino acid anhydride by the free ^{14}C-labelled amino acid liberated, leading to the precipitation of the corresponding poly-β-benzyl-aspartate derivatives.

Results similar to those recorded in Table 1 for β-benzyl-N-carboxy-L-aspartate anhydride were obtained when the N-carboxyanhydrides of the following amino acids were used as monomers: γ-benzyl-L-glutamate, ε-N-carbobenzoxy-L-lysine, phenylalanine, leucine, and methionine (see ref. 28). When t-RNA-leu-polytyrosine or t-RNA-ser-poly-ε-trifluoro-

TABLE 1—PRECIPITATION OF AMINOACYL RNA WITH β-BENZYL-N-CARBOXY-L-ASPARTATE ANHYDRIDE

[t-RNA was charged with ^{14}C-labelled serine, alanine or threonine, and the aminoacyl RNA formed was reacted with β-benzyl-N-carboxy-L-aspartate anhydride (*p*H 8·0). The precipitate formed was collected by centrifugation. The radioactivity in the precipitate was calculated from the difference in the radioactivity of the initial and supernatant solutions and the amount of RNA in the precipitate was determined from its phosphorus contents. The t-RNA in the initial and supernatant solutions was assayed for its amino acid acceptance. The amount of labelled amino acid in the precipitate was calculated from its radioactivity and RNA content[15]]

^{14}C-Amino acid bound to t-RNA	Fraction measured	Radio-activity c.p.m.	Amount of RNA μg.	Amino acid accepted Serine	Alanine	Threonine
				mμmole/mg. RNA		
Serine	Initial	2400	1320	1·9	1·5	1·5
	Supernatant	720	1240	0·7	1·6	1·5
	Precipitate	1680	80	22·5	..	..
Alanine	Initial	5460	1500	1·8	1·6	1·5
	Supernatant	2510	1433	1·8	0·7	1·4
	Precipitate	2950	67	..	20·0	..
Threonine	Initial	25500	4960	1·8	1·5	1·4
	Supernatant	7500	4783	1·8	1·4	0·8
	Precipitate	18000	177	..	..	27·5

acetyl lysine was prepared, the precipitates formed were found to non-specifically adsorb significant amounts of t-RNA from the reaction mixture.

Solubility of t-RNA polypeptides. The chemical nature of the polyamino acid attached to the t-RNA was found to markedly affect the solubility characteristics of the t-RNA-polypeptide derivatives isolated. This is shown in Table 2. In this connection it is pertinent to note that the fraction of aminoacyl RNA precipitated on adding N-carboxy-L-phenylalanine anhydride increases with increasing concentration of the anhydride from 5 to 25 mg./ml. This is most likely due to the formation of relatively long polyphenylalanine chains at high anhydride concentrations.

Recovery of biologically active t-RNA from polypeptidyl RNA. Preliminary studies indicated that the ester link between the ^{14}C-labelled amino acid and RNA in the various t-RNA-^{14}C-amino acid polypeptide derivatives isolated is somewhat more resistant at *p*H 10 than the same link in the corresponding aminoacyl t-RNA. These findings agree with the results of Gilbert[29] concerning the relatively high stability of biosynthetically formed polyphenylalanyl-RNA. They may also explain the presence of tightly bound peptides in t-RNA preparations[15,16]. The high stability of the polypeptide RNA to alkaline hydrolysis therefore prevents the liberation of fully biologically active t-RNA, since prolonged incubation of t-RNA at *p*H 10 causes considerable inactivation of its amino acid accepting ability.

TABLE 2—Solubility of t-RNA Polypeptides

Polypeptide attached to t-RNA	Solubility properties
Poly-γ-benzyl-L-glutamate	Soluble in DMF
Poly-β-benzyl-aspartate	Soluble in DMF
Poly-ϵ-carboxybenzyl-L-lysine	Soluble in DMF
Polyleucine	Insoluble in DMF, soluble in phenol
Polymethionine	Insoluble in DMF, slightly soluble in chloroform
Polyphenylalanine	Only soluble in concentrated H_2SO_4

It is interesting to note that positively charged polyamino acids seem to facilitate the hydrolysis of the ester link between the peptide and RNA. t-RNA-ser-^{14}C-polymethylsulfonium methionine bromide (derived from t-RNA-ser-polymethionine) and t-RNA-ser-^{14}C-polylysine (derived from t-RNA-ser-poly-ϵ-trifluoroacetylpolylysine), for example, yielded the corresponding serine labelled polypeptides at *p*H 8·5-9·0. However, the t-RNA liberated in both the cases possessed a low amino acid accepting ability. A similar observation was recently reported by Mehler and Bank[18]. At present some loss of activity accompanies the isolation procedure and further work is needed in order to correct it. It also remains to be rigorously proved that the material formed is, in fact, a polypeptide covalently linked to the amino group of the aminoacyl RNA initiator.

Purity of the t-RNA-AA-polypeptides. From Table 1 it can be seen that a highly enriched fraction can be obtained. The estimation of the final degree of purity of the RNA-AA-polypeptides depends on our precise knowledge of the molecular weight of t-RNA. Estimates of chain length of t-RNA have shown some variation. From physical and chemical studies the molecular weight has been estimated to be of the order of 25,000 to 30,000 (Ref. 30-32). There is also reason to believe that the different amino acid specific RNA chains do not differ significantly in their chain length[33]. Therefore, the approximate purity of the t-RNA-AA-polypeptides obtained is about 60%.

As mentioned earlier, at least in some cases, an amino acid specific RNA is "degenerated" and there are several different species capable of accepting the same amino acid. Our method will not differentiate between these species when a homologous enzyme is used. However, Keller and Anthony[34] using a rat liver enzyme and t-RNA from another organism (*Esch. coli*) showed that of the several types of leucine RNA known to be present, only one was charged with the amino acid. These results and earlier studies[25,35], indicate that by using a heterologous enzyme, our procedure may be applied to the separation of the "degenerate" forms of amino acid specific RNA. Alternatively, combination with another method such as countercurrent distribution could bring about the desired separation.

Some aspects of nucleotide sequence analysis of t-RNA. Most of the methods for the determination of the nucleotide sequence of amino acid specific RNA rely on the isolation and analysis of oligonucleotides produced by the action of specific nucleases. It has been shown that labelling the methylated bases in t-RNA can aid in the determination of the sequence analysis of such oligonucleotide fragments[36]. In these studies a methionine-requiring *Esch. coli* mutant $K_{12}W_6$ was grown on a defined salt medium containing ^{14}C-methyl-L-methionine and ^{32}P-orthophosphate. The t-RNA was isolated from these cells. All the ^{14}C-label incorporated was found in the methylated bases, while all the nucleotides were labelled with ^{32}P. The ^{14}C-^{32}P-labelled RNA was digested with RNase T_1 and the oligonucleotides resulting from the digestion of t-RNA were then fractionated into groups by chromatography on a DEAE cellulose column. Fig. 2 shows the results of the chromatography; it can be seen that the ^{14}C-containing oligonucleotides were resolved in a series of peaks. The first three ^{14}C peaks contained 5·1, 3·6 and 37% respectively of the ^{14}C-label. On further fractionation by paper electrophoresis (*p*H 3·5) and paper chromatography [twice in isobutyrate—0·5 *M* ammonia (10:6 vol./vol.)] and then in *tert*-butanol formate buffer, *p*H 3·8 (5:5 vol./vol.), at least 24 discrete components were resolved (six from peak 1, five from peak 2 and thirteen from peak 3). Thus, there are considerable numbers of oligonucleotides containing methylated bases in unfractionated t-RNA indicating that the individual t-RNA chains differ in the sequence of nucleotides around each methylated base. With a purified amino acid specific RNA the digest should be far less complex and more amenable to analysis since only a few ^{14}C-containing oligonucleotides will be present. The determination of their position would provide useful markers towards subsequent determination of nucleotide sequence.

A promising tool in the study of nucleotide sequences of t-RNA has recently evolved from the discovery[37] that t-RNA can be methylated *in vitro* by the enzymatic transfer of methyl group from s-adenosyl methionine[38,39]. Several RNA-methylases have already been purified. Thus, an *Esch. coli* extract contains at least five methylating enzymes among which there are two different guanine methylating enzymes both yielding l-methyl guanine residues[40]. This suggests that base sequence, and perhaps the secondary structure of the t-RNA chain as well, are responsible for the high degree of specificity of these enzymes. Moreover, it was found that RNA methylases are species specific and consequently t-RNA while fully saturated with respect to its homologous enzymes, offers new sites for methylation to a heterologous RNA methylase[40,41]. Once more, highly purified preparations of these enzymes become available. The resulting information concerning

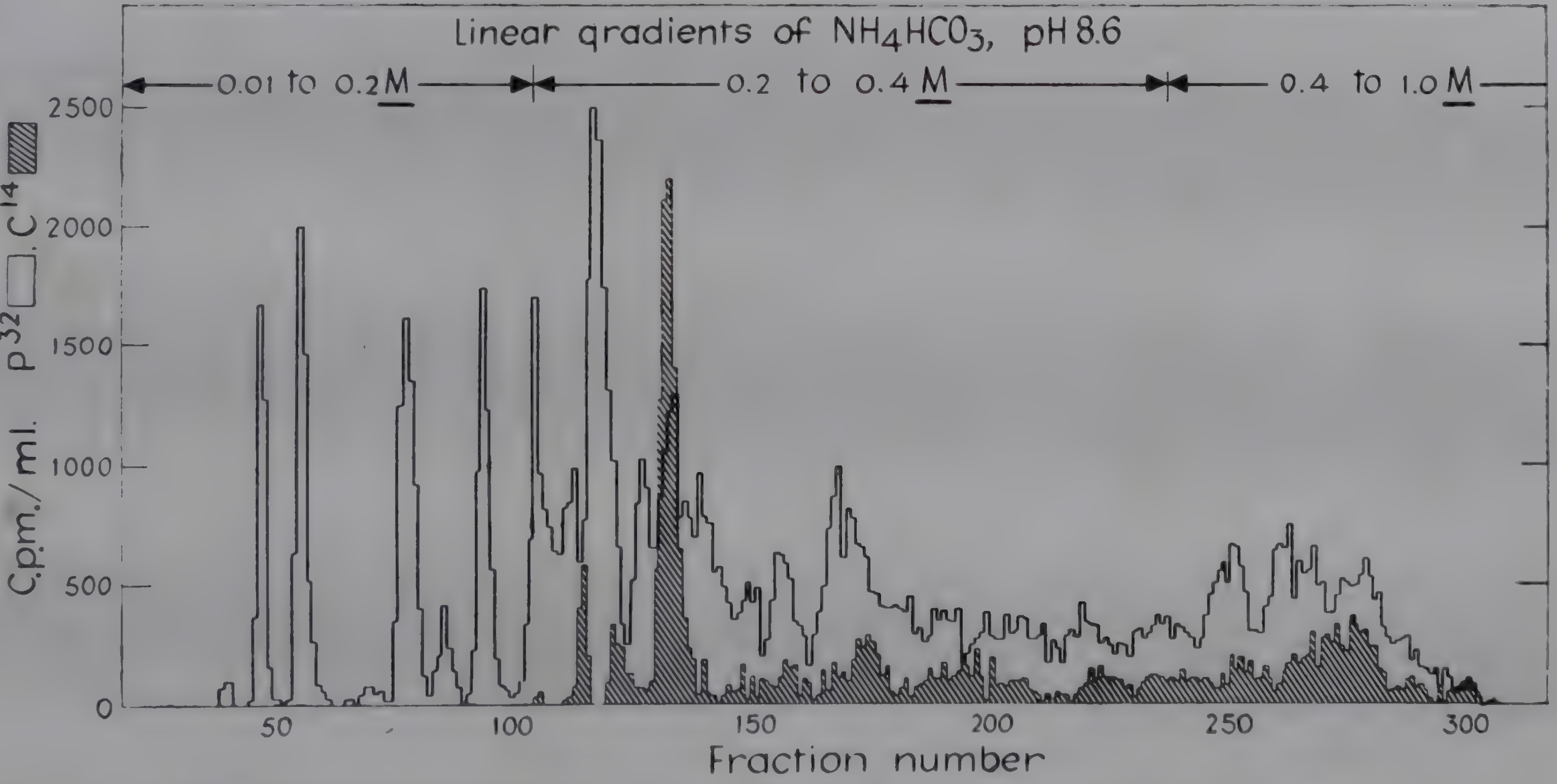


FIG. 2—CHROMATOGRAPHIC SEPARATION OF FRAGMENTS PRODUCED BY RNASE T_1 DIGESTION OF ^{14}C-METHYL, ^{32}P-AMINO ACID-ACCEPTOR RNA[36]

their specificity with respect to polynucleotide primary and secondary structure may well lead to the development of useful tools towards the identification of specific sequences in t-RNA chains.

CONCLUSION

It was suggested by Nathans and Lipmann[42] that a growing polypeptide chain is covalently linked to t-RNA at its carboxyl end. Gilbert[29] has recently isolated polyphenylalanyl t-RNA from a cell-free system of *Esch. coli* in which poly U was used as a messenger. Also, in the case of poly A directed synthesis, polylysyl-t-RNA was isolated[43]. The method described by us should prove to be a general one since as shown above, various polypeptides can be attached to t-RNA. Chemically synthesized t-RNA-polypeptides should resemble in their chemical and biological properties the corresponding compounds accumulated in a cell-free system induced by synthetic polynucleotides[44], and might be used as model compounds in the clarification of some of the intermediate stages of protein biosynthesis.

SUMMARY

A general procedure for the preparation of specific transfer RNA polypeptides has been introduced. t-RNA was esterified enzymatically with a single amino acid and the primary aliphatic amine of the aminoacyl RNA (RNA-AA) was used as initiator for the polymerization of a suitable N-carboxy α-amino acid anhydride, leading to the formation of RNA-AA polypeptide. By the preparation of water insoluble t-RNA-polyamino acid derivatives, a highly enriched preparation could be obtained. When β-benzyl-N-carboxyl-L-aspartate anhydride was used as monomer and the polymerization initiated with t-RNA-serine-^{14}C, t-RNA-alanine-^{14}C or t-RNA-threonine-^{14}C, the precipitate formed contained 50 to 70% of the initial counts and 4 to 6% of the initial t-RNA. Assuming a M.W. of 30,000 for t-RNA, the purity of the specific-AA-polypeptide fraction was about 60%.

The ester bond between the ^{14}C-labelled amino acid and RNA in the various t-RNA-^{14}C-amino acid polypeptide derivatives isolated, is more resistant to hydrolysis by alkali than the same link in the corresponding aminoacyl t-RNAs. The chemical nature of the polyamino acid attached to the t-RNA was found to markedly affect the solubility characteristics of the t-RNA-polypeptides isolated.

The use of chemically synthesized t-RNA-polypeptides as model compounds in the clarification of some intermediate stages of protein biosynthesis was discussed.

The determination of nucleotide sequence of specific transfer RNAs in conjunction with the distribution of methylated bases was also considered.

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A Model for the Base Sequence of Serine S-RNA

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S-RNA fulfills two very specific types of function: (1) *Acceptor* function of activated amino acids with formation of amino-acyl S-RNA; and (2) *donor* function of the amino-acyl moiety to a polypeptide chain in protein synthesis. The elucidation of the molecular basis of these functions has long been one of the principal research objectives in our own laboratory and in a number of other laboratories both in America and Europe. Furthermore, as is generally realized the correlation of chemical and physico-chemical data with biological function has been one of the long range goals in nucleic acid research for the last 20 years or so. From this point of view, S-RNA is a particularly suitable biological object since it is the smallest known nucleic acid and thus the task of determining its structure and base sequence and correlating the findings with biological function should be easier than for larger and more complex nucleic acids such as the RNA viruses.

In earlier work from my laboratory, in collaboration with McCully[1], we have shown that it is possible to construct a detailed base sequence for "average" rabbit liver S-RNA by correlating the results of physico-chemical studies with the partial base sequence data obtained through specific enzymatic cleavage of S-RNA with a number of endonucleases, such as pancreatic ribonuclease and the so-called T_1 ribonuclease. Our effort led to a structural model which permits a reasonable interpretation of what is known about the biological functions of S-RNA, and agrees well with physico-chemical data on S-RNA structure (Fig. 1). In order to build this type of hypothetical structural model it was necessary to make a number of assumptions. The same assumptions were used by Cantoni, Ishikura, Richards and Tanaka[21] to build an analogous model for serine specific S-RNA from yeast. It will be necessary, therefore, to review the background of these assumptions and assay their validity before going any further.

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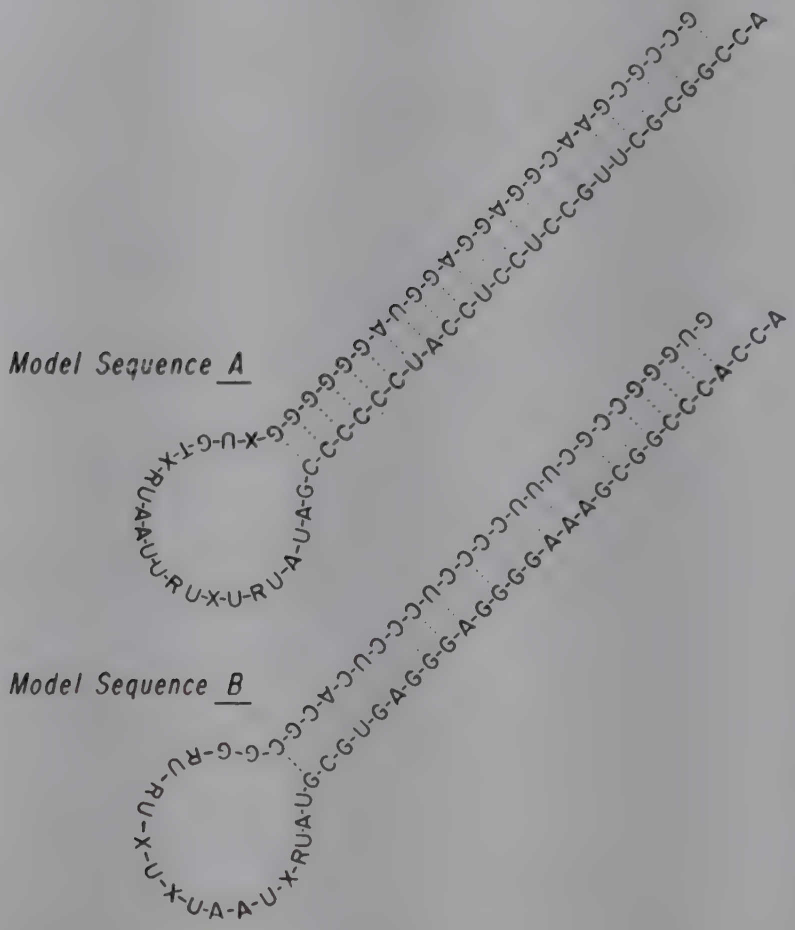

FIG. 1—TWO MODEL BASE SEQUENCES, A AND B, OF RABBIT LIVER S-RNA. CHAIN LENGTH 70: The average of the two sequences approximates the base composition data and the base sequence frequency data. The area where the bases are paired specifically is in the double-helical configuration[12]. This pair of model sequences is not unique since other pairs of sequences can be found that agree with the assumptions stated in the text and the experimental data [From McCully, K. S. & Cantoni, G. L., *J. mol. Biol.*, **5** (1962), 497]

There is one principal assumption and two corollary assumptions. The principal assumption requires that there be a maximum of contiguous specific base pairing consistent with the frequency data. Clearly the configuration which permits the greatest number of contiguous base pairs is for the polynucleotide chain to be doubled back upon itself to form a double helix between the two limbs. This assumption has the effect of reducing very drastically the number of oligonucleotide fragments required to define a unique base sequence. Moreover, if one keeps in mind the fact that it is for the time being technically impossible (or at least very difficult) to obtain from RNA large and specific oligonucleotide fragments with which one could do for S-RNA what Sanger did so elegantly with the oligopeptides derived from insulin you can appreciate how useful this hypothesis will turn out to be if proved correct.

The assumption that S-RNA contains a single, long, helical region exhibiting Watson-Crick[2] base pairing and complimentary sequences, comes from three lines of experimentation: physical, chemical and enzymatic.

The physical studies show that when S-RNA is heated in dilute salt solution at neutral *p*H it exhibits a marked hyperchromicity. The degree of hyperchromicity permits an estimation of the proportion of bases which exist in the double helical configuration. There have been many studies of the temperature dependent hyperchromicity of S-RNA, and a number of estimates of the percentage of base pairing in S-RNA. In the most recent study, Felsenfeld and Sandeen[3] reached the conclusion that if one excludes the methylated components ψ UMP and the PCpCpA terminal sequence, which, as you know, is believed to be common to all the different amino acid specific S-RNAs, all or nearly all the G-C and the A-U pairs which can be anticipated from the base composition are present in S-RNA. Somewhat similar estimations have been reached earlier by Cox and Littauer[4], Brown and Zubay[5], and Fresco and his collaborators[6], so that it is generally agreed from the data on temperature denaturation that a very large fraction of the bases in S-RNA exists in double helical configuration.

More direct evidence that there is only *one* such base paired region comes from a study of the relationship between the increase in optical density of a solution of S-RNA and the degree of hydrolysis with snake venom phosphodiesterase[7]. As it is well known, this enzyme cleaves ribonucleotides stepwise from the end that bears the 3′-hydroxyl group[8-10]. With Nihei we have shown that when S-RNA is digested with snake venom phosphodiesterase the hyperchromic effect develops more rapidly and approaches completion with 40-50% of the polynucleotide chains being digested to mononucleotide residues. On the other hand, in a similar experiment in which we used 0·3 *N* KOH to produce random endonucleolytic cleavage, we observed a hyperchromic effect that more or less paralleled the degree of hydrolysis (and in fact exhibited a small initial lag) (Fig. 2). I think this kind of experimental result is best compatible with the presence of a single double stranded region, although minor imperfections in the continuity of the base paired region such as have been proposed by Fresco[6] would not have been detected. Such small loops consisting of one or two unpaired bases would not have a significant effect on the general features of the structural model proposed. Different types of models containing two or more separate helical regions can be, and have been, proposed (Fig. 3). In these models, however, there should be a fairly good correlation between the degree of hydrolysis and the degree of hyperchromicity.

Ultracentrifugation and viscosity studies of S-RNA[11] also contribute support to the concept that S-RNA has a DNA-like structure, since these studies indicate that the S-RNA molecule exhibits hydrodynamic asymmetry, the axial ratio for unhydrated equivalent ellipsoids being close to 9 : 1 which is not too different from an axial ratio of 6: 1 which has been calculated from Fuller's data[12] for a DNA-like helical chain of 35 base pairs. Studies of S-RNA structure with the electron microscope, although far from satisfactory also support the notion that S-RNA molecules are like structures 1000 A. long and 20 A. wide[12a].

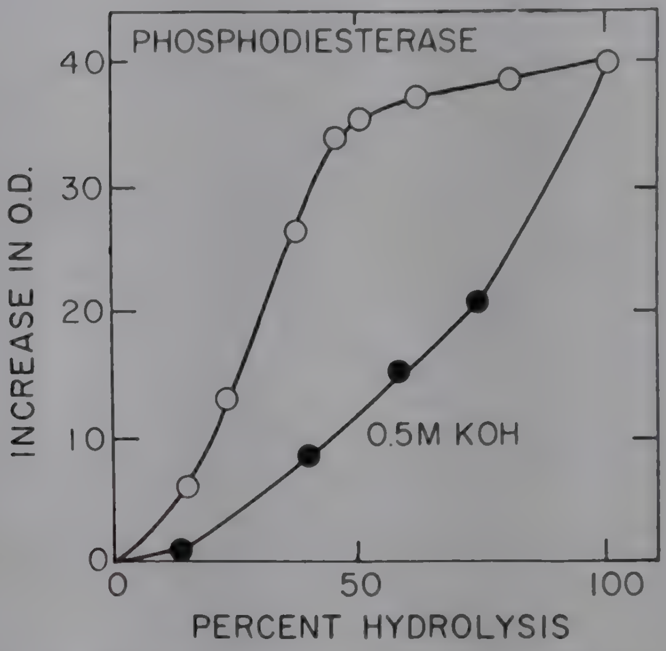

FIG. 2—CORRELATION BETWEEN HYPERCHROMICITY AND DEGREE OF HYDROLYSIS: O, hydrolysis by venom phosphodiesterase; ●, hydrolysis by 0·4 *M* KOH at 37° [From Nihei, T. & Cantoni, G. L., *J. biol. Chem.*, **238** (1963), 3991]

An independent line of evidence suggesting base pairing comes from chemical studies which indicate good statistical agreement between the number of adenine and uracil or guanine and cytosine residues in average S-RNA. This is a somewhat weaker argument because we must anticipate that in purified S-RNA there will be some deviation from the precise pattern which is observed in average S-RNA, due to the presence of different amounts of methylated purines, pyrimidines, and, perhaps ψ-uridine and, in addition, to the presence of a coding triplet complementary in its base composition to the "codon" in messenger RNA. More important are the studies of Pennington and Doty[12b], who, on the basis of their elegant studies of the interaction of S-RNA and formaldehyde, have reached the conclusion that only 14% of the bases in S-RNA are unpaired.

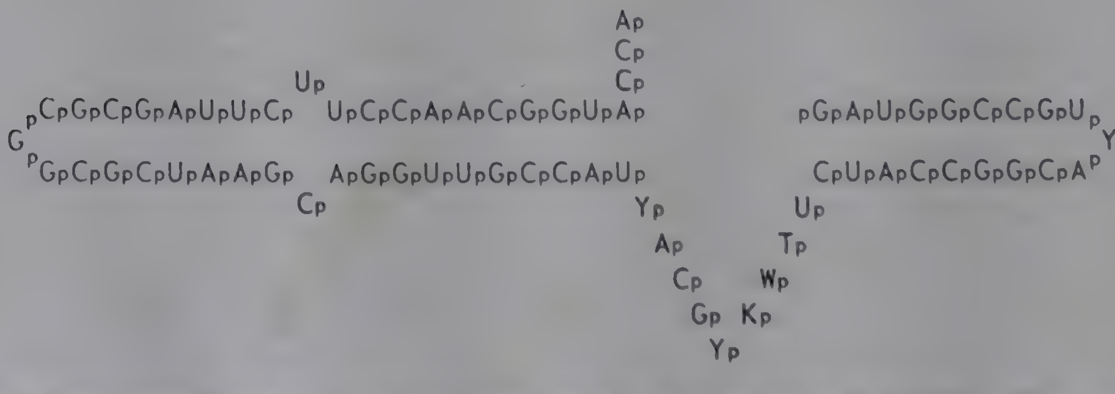
Ap
Cp
Cp
Up
pCpGpCpGpApUpUpCp
UpCpCpApApCpGpGpUpAp
pGpApUpGpGpCpCpGpUp
G
Y
pGpCpGpCpUpApApGp
ApGpGpUpUpGpCpCpApUp
CpUpApCpCpGpGpCpAp
Cp
Yp
Up
Ap
Tp
Cp
Wp
Gp
Kp
Yp

FIG. 3—ALTERNATIVE AND HYPOTHETICAL BASE SEQUENCE MODEL OF S-RNA: This model differs from that shown in Fig. 1 by exhibiting two base paired regions, imperfect base pairing and looping out

A series of enzymatic studies have shown that S-RNA has unusual resistance to a variety of nucleases and to other enzymes capable of degrading nucleic acids. This was originally shown in studies by Marianne Grunberg-Manago[13] and by Singer, Luborsky and myself[14], on the resistance of S-RNA to polynucleotide phosphorylase; a number of later observations[15-17] have also shown that S-RNA is resistant to the action of pancreatic and T_1 ribonuclease, and to *B. subtilis* ribonuclease, snake venom phosphodiesterase, etc. These results and the temperature dependence of the hybridization of S-RNA with heat denatured DNA are certainly related to the secondary structure of S-RNA, although it is hard to evaluate quantitatively the helical content of S-RNA molecules from these studies alone.

The principle of maximum contiguous base-pairing logically leads to a corollary assumption which states: the methylated purines and pyrimidines and ψ-uridine are clustered in the centre of the chain. Indirect evidence in favour of this assumption comes from observations of McCully and Cantoni[18] and of Staehelin[19] that the minor components in S-RNA tend to cluster together; and direct evidence was obtained from the study which Nihei and Cantoni[7,20] did on the distribution of the minor components after hydrolysis of S-RNA with snake venom.

The results of these experiments are shown in Tables 1 and 2.

Here we digested S-RNA with snake venom phosphodiesterase and determined the amounts of the various mononucleotides formed at various degrees of hydrolysis. In experiment A, S-RNA was digested to 19% hydrolysis; determination of the amounts of CMP, GMP, and AMP and UMP formed reveals that they are present in approximately the same percentage of the total as the percentage digested. On the other hand, the fraction of ψ UMP, methylated GMP, and methylated AMP, found is much smaller percentagewise than the degree of digestion; in experiment B, where 77% of the chains had been demolished, you notice again that CMP, AMP and GMP are present in the digest in approximately the same amount, percentagewise, as the average overall digestion; ψ UMP and the methylated components, on the other hand, have been completely degraded to mononucleotides suggesting that in the intact average S-RNA chains these components are distributed between the 14th and 50th nucleotides (±8) and that, therefore, the terminal portion of the nucleotide chain is essentially free of these components. We have done many experiments of this type, and we are quite sure that there are no methylated components or ψ UMP in the first 30% of the chain or so. It is, however, very much more difficult to be equally sure that the unusual nucleotides are absent from the last third of the chain because, in this case, the values can only be obtained by difference rather than directly. It would be much safer and better if we could find a way to obtain the information about the last third of the molecule directly by digestion with an exonuclease, like the spleen phosphodiesterase, capable of removing nucleotides in stepwise fashion starting from the 5′-phosphate end; however, for the present at least, contamination with endonucleases makes the experiment impossible; it is not even certain whether a endonuclease-free preparation of spleen phosphodiesterase can be obtained.

TABLE 1—DISTRIBUTION OF MINOR COMPONENTS AFTER HYDROLYSIS OF S-RNA WITH SNAKE VENOM

"Region" number	AU/GC ratio in Region	Approximate number of		"Region" description	Approximate number of	
		AU	GC		AU	GC
1	1·9	4	2	mixed AU-GC region near loop	4	2
2	1·0	6 or 7	6	mixed AU-GC region near open end	7	7
3	~0·1	0 or 1	8	central pure G-C region	0	7

TABLE 2—BASE COMPOSITION OF AMINO ACID ACCEPTOR LIMB (A), NON-ACCEPTOR LIMB (C), AND CENTRAL SEGMENT (B) OF RABBIT LIVER S-RNA

	A (1-14)*	B (15-53)*	C (54-70)
	μmoles/μmole of S-RNA		
CMP+MeCMP	4·4	9·3	6·5
AMP	2·8	6·8	2·5
GMP	3·4	12·8	5·3
UMP	2·2	7·3	0·3
5-RUMP	<0·1	2·1	0·1
MeGMP	0·1	1·1	0·1
MeAMP	<0·05	0·4	0·05
X_1MP+X_2MP	0·4	1·4	0·1
TOTAL	13·35	41·2	14·95

*Number in parenthesis refer to nucleotides from amino acid acceptor end.

A second corollary follows from the principal assumption requiring maximum base pairing, namely that the terminal nucleotide be paired with position 3 of the acceptor limb. Base pairing could also begin with the first cytidine residue, but, in fact, in some of the models this would have resulted in some complications and reduced base pairing possibilities. Moreover, there is some indirect evidence in favour of this idea from the work of Preiss *et al.*[21] and Furth *et al.*[22] on the properties of the so-called CCA pyrophosphorylase. These authors have shown that when S-RNA is cleaved by this enzyme it loses quite easily the terminal adenosine, and one of the two terminal cytidylic acid residues. The second cytidylic acid residue is very resistant to the action of this enzyme suggesting that there is some impediment to its removal. A hydrogen bond might provide such an impediment, as might the 5′-phosphate of the terminal GMP.

The model which results from these assumptions and hypotheses suggests that the structure of S-RNA consists of a double-stranded helical region of about 25 base pairs and a loop, which contains the methylated bases, of which there may be one or several in an S-RNA molecule*, the 3 ψ UMP residues and the triplet which reacts with the messenger RNA, which triplet we might call "nodoc" (which is codon spelled backwards).

Certain conclusions as to the relationship between structure and function follow rather directly from this type of model. Naturally, these conclusions are just as hypothetical as the model is and if the model turns out to be wrong the conclusions also will turn out to be wrong. It is clear from what is known about the biological and biochemical properties of S-RNA that each molecule must have two functional sites, one for *acceptor specificity*, and the other for *transfer specificity*. The *acceptor specificity* is the site which would interact with the amino acid activating enzyme; the *transfer specificity* is the site which reacts with the messenger.

Crick[25], as far back as 1958, recognized the need for the *transfer specificity* site to be single-stranded (or at least not double-stranded in the regular Watson-Crick fashion); this prediction agrees well with the finding of Nirenberg and Matthaei[26] that the polyuridylic acid is active in the S-RNA dependent synthesis of polyphenylalanine, only when it is in the single stranded conformation, and with more recent findings of Takanami and Okamoto[26a] on the inhibitory effect of secondary structure on the biological messenger-like activity of synthetic polynucleotides†. It has been pointed out both by Cantoni[27] and by Zamecnik[28], that the presence of the methyl group in methylated purines and pyrimidines affects the base pairing potentialities in such a way as to decrease or make it impossible in some cases to have base pairing so that clustering of the methylated bases together would automatically provide a region in which it is difficult to have the regular Watson-Crick base pairing. This "loop" region does not necessarily need to be single-stranded; it can be base paired in some non-Watson-Crick form, or in an irregular and less stable manner.

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*In serine S-RNA there is only one[23], but in alanine S-RNA there are as many as 11 according to Holley[24].

†In natural messenger, however, the structure may be more coupled, as it is known that RNA virus exhibits a considerable degree of secondary structure.

One of the fragments that we have identified in serine specific S-RNA has the composition, PypApGpAp ψ Up. Since this fragment contains ψ UMP we have decided that it originated from the so-called "loop." A second ψ UMP can hypothetically and speculatively be assigned to represent the pyrimidine which begins the sequence so that the sequence can be represented as ψ UpApGpAp ψ Up. AGA are the complimentary bases to triplet UCU, the three letters representing one of the "codons" for serine (the order of the letters in the "codon" not being known at the present time). Tentatively, therefore, we can assign to ψ UMP the function to punctuate or locate the position of the "nodoc" in the S-RNA chain. ψ UMP would be particularly well suited chemically for such a role since it can form twice as many hydrogen bonds as UMP and also since it can form hydrogen bonds not only with AMP, but with CMP and GMP as well.

Assuming that the two functional sites are separate, one might postulate that the double-stranded region would contain the *acceptor specificity* site. One advantage of this arrangement would be that a relatively well-defined tridimensional surface of the type which is usually associated with ligand-protein combination would be available for the reaction between S-RNA and the specific amino acid synthetases. Other models are possible and it might be helpful to keep in mind that the molecular size of the amino acid activating enzyme is quite large. We have estimated with Makman that the molecular weight of the serine activating enzyme from yeast may be of the order of 100,000 or so, and, therefore, more than large enough to envelope a very large part of the S-RNA molecule. There are three lines of evidence which suggest that the amino acid acceptor site and the transfer specificity site are separate. In the first place, there is the work of Arca *et al.*[29] who have shown that S-RNA is no longer able to accept activated amino acids under conditions where the secondary structure of S-RNA is lost due to thermal or chemical denaturation. Next, there are the experiments of Berg *et al.*[30] showing that important base sequence differences occur in this portion of the limb for various amino acid specific S-RNAs. And finally, there are the very interesting recent experiments of Ebel and his collaborators[31], and of Yu and Zamecnik[32] on the effect of chemical alteration of S-RNA, bromination or methylation, on its amino acid acceptor activity. Specifically, in a very elegant experiment, Ebel was able to correlate the type and degree of chemical modification to loss of biological acceptor function: under conditions of bromination, where none of the uridine residues had been modified, he observed complete loss of acceptor activity for lysine, and only approximately 50% loss for phenylalanine. As you know, bromination does not affect the adenine

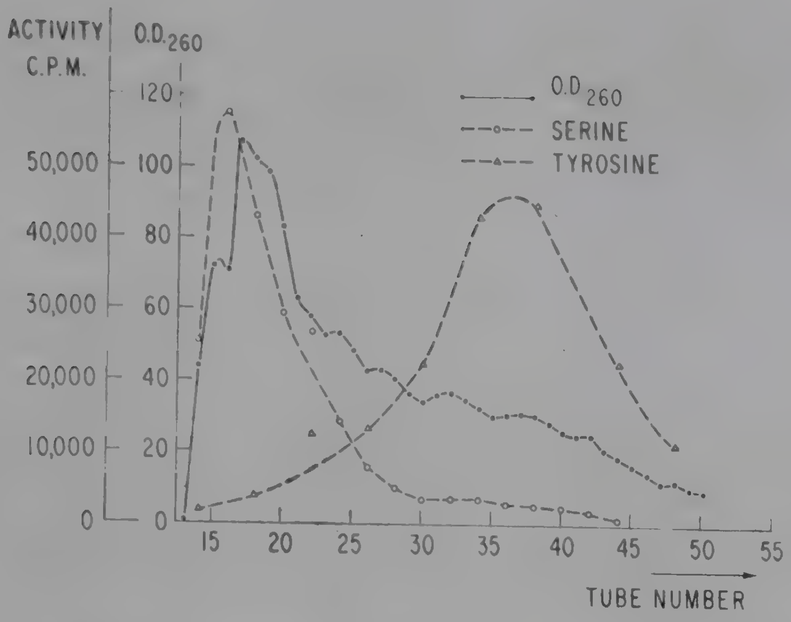

FIG. 4—PARTITION CHROMATOGRAPHIC SEPARATION OF YEAST S-RNA: The amino acid acceptor activities are plotted as total counts per minute per fraction. Absorbancy at 260 mμ (O. D.$_{260}$) also is plotted as total absorbancy per fraction [From Cantoni, G. L., Ishikura, H., Richards, H. H. & Tanaka, K., *Cold Spr. Harb. Symp. quant. Biol.*, **28** (1963)]

residues. Thus, if we accept the assumption that the transfer specificity site in S-RNA contains a triplet with a base composition complementary to that of the "codon", it might be tentatively concluded that the amino acid acceptor function in this chemically modified S-RNA is not related to the structure of the "nodoc." In the case of lysine, we would expect the "nodoc" to be UUU; in case of phenylalanine it would be AAA and, as you appreciate, in one case it would be affected very much by the bromination and in the other case not at all, in keeping with the idea that there are two separate sites in the S-RNA molecule for these two functions.

Before describing the base sequence model for serine S-RNA, I would like to discuss very briefly the preparation and some of the properties of serine S-RNA. In collaboration with Tanaka *et al.*[33] we have shown that serine S-RNA can be obtained in highly purified form by partition chromatography on Sephadex using Zachau's solvent system[34]. Chromatography on Sephadex is particularly advantageous because it permits large scale fractionation with relative ease. Fig. 4 shows the results of partition chromatography of average yeast S-RNA on Sephadex. As you see, we obtained a reasonably good separation of S-RNAs specific for serine, tyrosine, leucine, threonine and valine. We have also located the position of other S-RNAs specific for the remaining amino acids.

Fig. 5 shows the results of rechromatography of the peak enriched in serine S-RNA; in this figure are plotted the optical density profile, the specific activity and the total activity. It can be seen that the total activity follows closely the total optical density profile and that the specific activity remains relatively constant suggesting reasonable purity in the material. On a third

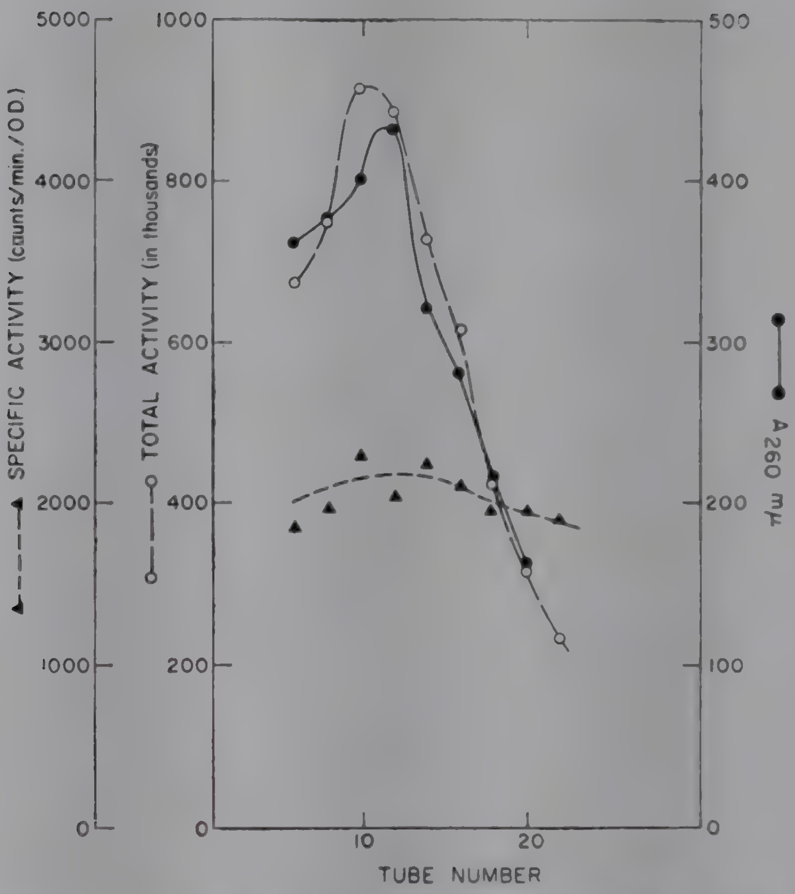

Fig. 5—Rechromatography of serine S-RNA peak from Fig. 2: The solvent system contained 5,500 ml. of solution A (10 litres butanol, 1 litre redistilled tri-N-butylamine): 6,625 ml. of solution B (13 litres H_2O, 250 ml. glacial acetic acid); and 1,200 ml. of N-tributyl ether. The system was equilibrated at 23-24°C. Column size, 3 × 140 cm.; fraction size, 5 ml. [From Cantoni, G. L., Ishikura, H., Richards, H. H. & Tanaka, K., *Cold Spr. Harb. Symp. quant. Biol.*, **28** (1963)]

rechromatography, we obtained a more symmetrical peak and still better agreement. The serine S-RNA which we obtain in this way has been tested as an acceptor of the other 19 amino acids, and it exhibits better than 98% specificity for serine. Also, under favourable conditions we can demonstrate the incorporation of nearly 1 μmole of serine/μmole of S-RNA on the basis of a chain length of 70. There is one important reservation that we must keep in mind at this point about the purity of the material, namely, that on the basis of amino acid acceptor function alone it is impossible to differentiate between two or more degenerate species of serine S-RNA, since presumably they would all function as substrates for the same activating enzyme.

Table 3 shows a comparison between the composition of serine S-RNA and that with average yeast S-RNA. In yeast S-RNA, there are a variety of methylated purines (6-methylaminopurine, 2-dimethylguanine, 1-methylguanine), each present in amounts smaller than one residue per chain indicating that some chains must contain at least one residue per chain, while in other chains one or several of the methylated purines are missing. In the serine S-RNA, on the other hand, there is only one methylated

TABLE 3—Base Composition of Average Yeast S-RNA and Serine S-RNA

Yeast S-RNA		Serine S-RNA	
CMP	19·37±0·5	CMP	16·1±0·8
AMP	12·71±0·1	AMP	14·1±0·4
6MeAMP	0·38±0·05	diMeGMP	1·0±0·15
diMeGMP	0·67±0·17	ϕUMP	3·0±0·2
1′MeGMP	0·66±0·25	GMP	19·6±0·3
ϕUMP	2·72±0·4	UMP	14·4±0·9
GMP	19·10±0·4	TrMP	1·0
UMP	12·37±0·2	X	0·75
TrMP	0·7 ±0·5		
Total	70	Total	70

The nucleoside and nucleotide composition was determined by separation of the product of alkaline hydrolysis[15]. The chain length of the S-RNA is 70.

purine, 2-dimethylguanine, and the amount is one residue per chain; there is no 6-methylaminopurine and no 1-methylguanine. There are also three ψ-uridylic acid residues and 1-thymine ribotide residue per chain, and perhaps one 5-methylcytidine residue per chain.

Another point which stands out is that while in average S-RNA there is quite good agreement between CMP and GMP, and AMP and UMP, in serine S-RNA there is good agreement between AMP and UMP, but there is an excess of GMP over CMP residues.

We have proceeded to hydrolyse serine S-RNA by use of the following specific endonucleases: Takadiastase streptomyces[35] and pancreatic ribonucleases. After characterization of the various fragments by column chromatography we attempted to put these pieces together following the rules or assumptions which I have outlined earlier. The results of our work on this wonderful puzzle is shown in Fig. 6.

I must emphasize that it is only a tentative *model* of the total sequence of serine S-RNA. You recognize the...CpCpA and the...pGp ending. The direction of the chain in these two limbs, of course, is anti-parallel and this is shown by the two arrows. The white line underlines the number of partial sequences which have been identified by the techniques which I have just outlined. The dots refer to ambiguity in our base assignments. The bases in all such uncertain assignments are pyrimidines and they correspond to the left hand neighbours of fragments obtained by pancreatic

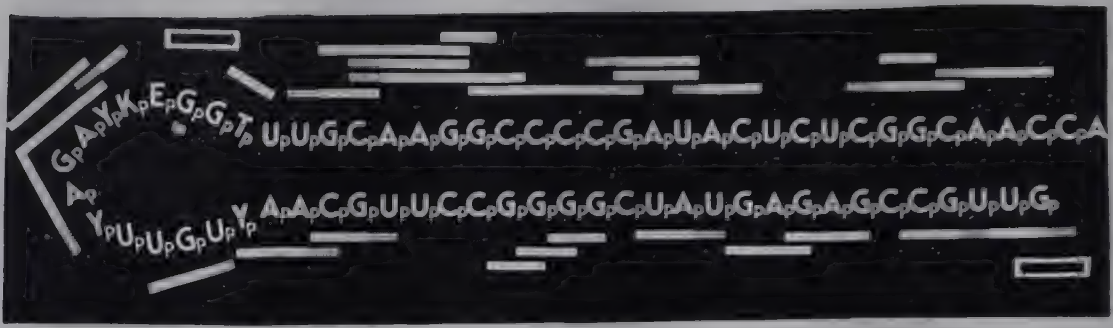
GpApYpKpEpGpGpTp UpUpGpCpApApGpGpCpCpCpCpGpApUpApCpUpCpUpCpGpGpCpApApCpCpA
Ap
YpUpUpGpUpYp ApApCpGpUpUpCpCpGpGpGpGpCpUpApUpGpApGpApGpCpCpGpUpUpGp

FIG. 6—MODEL BASE SEQUENCE OF YEAST SERINE S-RNA: G, guanosine; C, cytidine; A, adenosine; U, uridine; Y, 5-ribosyluridine; K, 2-dimethylaminoguanosine; E, unknown nucleoside (alkali resistant). The polarity of the base sequence in the two lines is opposite. In lower line, p at the right of base symbol is 5′-phosphate, in upper line, p at the right of base symbol is 3′-phosphate. Identified partial sequences are represented by white lines under (or above) nucleotide sequence. Ambiguity in identified partial sequences is indicated by dotted portion of white lines under (or above) nucleotide sequence [From Cantoni, G. L., Ishikura, H., Richards, H. H. & Tanaka, K., *Cold Spr. Harb. Symp. quant. Biol.*, **28** (1963)]

ribonuclease digestion. It is of interest that there are some areas of overlap; the partial sequences involved are made to overlap in this type of reconstruction, but these overlapping sequences are not true overlaps in the sense of the protein chemists. The experimentally identified partial sequences, not including the short sequences pyrimidine C and pyrimidine U, account for at least 59 out of the 70 nucleotides in serine S-RNA. There are, therefore, 11 nucleotides which we have placed more or less at random.

The data so far obtained are not sufficient to define a unique base sequence. I do not know yet just how many other sequences we can make, and we are in the process of attempting to compute this. Intuitively, I felt that although a fairly large number of different base sequence models may be assembled from the available data, they would fall in quite a small number of families of models; the results of experiments on thermal denaturation performed with Felsenfeld that will be discussed presently support this notion. I think we will need some additional and perhaps different oligonucleotide fragments before we can choose between the variety of similar base sequence models that are possible at the present time. As noted earlier the sequence, ψ UpApGpAp ψ Up, has been placed in the centre of the loop; we have also placed there the methylated purine and thymine. I should point out that the sequence assignment in this region is very much more tentative than in the double-stranded region. It may be noted that the proposed model base sequence shows the presence of a reasonably long region, in the centre of the molecule, which is pure GC. This is due to the fact that we have identified provisionally one large fragment, GpCpCpCpCpGp, and, therefore, on the base pairing assumption we placed the corresponding sequence, CpGpGpGpGpCp, on the other limb.

TABLE 4—Base Composition of Independently Melting Units in Serine S-RNA

Unit	Composition predicted from multiwave length analysis	Model base sequence
Pure GC	8 GC pairs	7 GC pairs
Mixed AU and GC	$\frac{AU}{GC}$ 2·6	2·0
Mixed AU and GC	$\frac{AU}{GC}$ 1·0	1·0

TABLE 5—Base Composition of Average and Serine-rich S-RNA of *Esch. coli* B

Average S-RNA		Serine-rich S-RNA	
CMP	21·0	CMP	19·6 ±0·15
AMP	11·8	AMP	14·45±0·05
GMP	19·2	GMP	19·80±0·00
UMP	11·4	UMP	10·77±0·23
ψUMP	2·2	ψUMP	1·90±0·30
diMeGMP	<0·10	diMeGMP	<0·10
6 MeAMP	0·10	6 MeAMP	<0·10
7 MeGMP	0·95	7 MeGMP	<0·10
TrMP	0·69	TrMP	0·93±0·40
X	1·0	X	1·00
Total	70	Total	70

The nucleoside and nucleotide composition was determined by separation of the product of alkaline hydrolysis[15]. The chain length of the S-RNA is 70.

In collaboration with Felsenfeld (in press) we have recently obtained by an independent approach good evidence which supports the general distribution of GC and AU pairs shown in Fig. 6. The experiments with Felsenfeld are based on multiwave-length denaturation analysis; this technique allows separate estimation of the degree of denaturation of the AU and GC pairs as a function of temperature; therefore, under favourable conditions it can give a rather precise estimation of the base composition of independently melting units. With this technique, Felsenfeld arrived at the conclusion that serine S-RNA must contain one region comprising one-half of all the GC pairs and no AU pairs (or 8 GC pairs), and at least two other different regions containing AU pairs and GC pairs in different ratios. In one of these regions we would expect to find that the AU pairs are in excess of the GC pairs by 2·6 to 1 ratio; in the other region we anticipate near equivalence of AU to GC pairs. Table 4 shows that there is excellent agreement in the base pairs distribution anticipated from the multiwave-length denaturation analysis and that proposed in the model base sequence from purely chemical data. The assumptions used in the two experimental approaches are different and, therefore, the agreement is all the more striking and convincing.

In addition to the special problems relative to the definition of a unique base sequence for serine specific and other amino acid specific S-RNAs there remain, however, a number of interesting features of the structure of S-RNA which at the present time remain quite puzzling and which in the long run must be answered if we are to fully understand the chemistry of S-RNA. One of these relates to the function of the methylated bases in S-RNA. When we found that there is only one methylated purine in serine S-RNA we were rather pleased and hopeful that perhaps one could arrive at some meaningful relationship between the methylated bases and the amino acid specificity. For this reason we decided to examine the base composition of serine S-RNA from *Esch. coli* to see whether it also contained only one dimethylguanine per chain. With Nathenson (in preparation), however, we found (Table 5) that serine specific S-RNA from *Esch. coli* does not contain 2-dimethylguanine so that we cannot with safety ascribe to dimethylguanine a function directly related to the specificity for serine. The role of methylated components of S-RNA must remain for the time being one of the unanswered questions in the molecular biology of S-RNA.

In conclusion, I would like to say that we feel reasonably confident that the model which I have shown you is a close facsimile of the actual structure of serine S-RNA. I do not claim that it is *the* sequence for serine S-RNA, but I would hope that when it is finally known, the base sequence of serine S-RNA will turn out to have a very close resemblance to the one which we are now proposing.

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Studies on Mutants and Strains of Tobacco Mosaic Virus

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Tobacco mosaic virus (TMV) is a rod-shaped particle with a length of 3000 A. and a diameter of 150 A. It consists of an RNA helix, which is surrounded by a protein coat made up of 2200 identical subunits.

Alterations in the genetic substance of the virus, its RNA, can lead to mutants which have amino acid exchanges in the viral coat protein. Therefore, it is possible to study the effect of a specific alteration within the RNA on the amino acid sequence of the protein which is coded by this RNA, i.e. to obtain information about the genetic coding problem.

Furthermore, from studies on mutants and strains conclusions about the fine structure of the virus can be drawn.

METHODS

Our investigations on the genetic code are carried out as follows: Isolated TMV-RNA or the intact virus is treated with specific mutagenic agents whose action is known. Mutants resulting from this treatment are isolated and grown in larger quantities. The virus is then isolated from infected plant leaves and split into its RNA and protein moieties. The protein is digested with trypsin, and the tryptic peptides are isolated, purified and hydrolysed. The amino acid composition of each peptide is quantitatively determined with amino acid analysers and compared with the corresponding peptide of the parent strain. Where amino acid differences are observed, the exact positions of the amino acid exchanges are located using the methods of sequence analysis. From the results obtained on a large number of chemically induced mutants, conclusions regarding properties of the genetic code may be drawn. Similar studies have been done by Tsugita and Fraenkel-Conrat[1] and Tsugita[2].

We have employed three different mutagens for our investigations: nitrous acid, hydroxylamine and 5-fluorouracil. The action of each of these agents of TMV-RNA has been previously described and may be summarized briefly as follows: (1) Nitrous acid—The mutagenic base conversions which are produced by treatment with nitrous acid are cytosine→uracil as well as adenine→(hypoxanthine)→guanine[3–5]. (2) Hydroxylamine—The specificity of this reaction is highly *p*H dependent[6–8]. At *p*H 6 cytosine is converted to compounds which behave as uracil analogues during base pairing. At *p*H 9 the reaction is mainly with uracil leading to a splitting of the pyrimidine ring. A mutagenic effect could be demonstrated with TMV only at *p*H 6 (i.e. C→U) and not at *p*H 9 (Ref. 10). (3) 5-Fluorouracil—If uracil in TMV-RNA is replaced by 5-fluorouracil (5-FU) during synthesis of TMV, then mutations are formed[11] probably because 5-FU occasionally pairs with guanine instead of adenine. One would, therefore, expect to find base conversions of U→C in the RNA. If the substitution of 5-FU occurs in the strand which is complementary to the infecting strand, then an exchange of A→G should appear in the strand which is incorporated into the virus.

Results and Discussion

In Table 1 is presented the number of mutants whose tryptic peptides were investigated for each of the individual mutagenic agents. The distribution of amino acid exchanges is for the nitrous acid mutants in good agreement with a Poisson distribution.

As shown in Table 1, only part of the mutants investigated have amino acid exchanges, although those mutants which do not differ in protein from the wild strain often exhibit marked differences in symptom formation. This finding led previously[12] to the conclusion, that only part of the TMV-RNA strand is necessary for coding of the viral coat protein. Amino acid exchanges in the coat protein can arise only when changes in nucleotides within this cistron occur. Alterations in nucleotides in other positions of the TMV-RNA can lead to a change in symptoms, perhaps through changes in enzymes whose production is controlled by the TMV-RNA, but not to changes in the coat protein.

TABLE 1—Number of Mutants whose Proteins have been Analyzed

Exchanges	Nitrous acid	Hydroxylamine	Spontaneous	5-FU
0	112	5	8	8
1	35	1	6	3
2	7	0	1	0
3	0	0	1	0
>3	0	0	0	0
Sum	154	6	16	11

The size of the cistron that codes the 158 amino acids of the virus coat protein can be estimated from the average number of deamination per RNA strand and the average number of amino acid exchanges. It is found that this cistron contains about 310 nucleotides[13]. Unfortunately, this estimation contains a degree of uncertainty because of selection factors which must be taken into account in making the calculation.

In Table 2 are presented the amino acid exchanges found along with the number of the tryptic peptide in which each exchange occurs. If the amino acid exchanges found in nitrous acid mutants are compared

TABLE 2—AMINO ACID EXCHANGES IN CHEMICALLY-INDUCED TMV MUTANTS

Exchange	Peptide	Times observed
	Nitrous acid	
Thr → Ileu	I	2
,, → ,,	III	2
,, → ,,	X	4
,, → ,,	XI	1
,, → Met	VIII	3
Ser → Phe	I	1
,, → ,,	XI	3
,, → Leu	I	1
,, → ,,	III	1
Pro → Leu	I	1
,, → ,,	XII	3
,, → Ser	IV	4
Ileu → Val	I	2
,, → ,,	X	2
,, → Met	I	2
AspN → Ser	VI	1
,, → ,,	XI	1
Asp* → ,,	I	2
,, → Gly	I	2
Asp → ,,	IV	2
,, → Ala	I	4
Leu → Phe	I	1
Glu → Gly	VIII	1
Glu* → Val	I	2
	5-Fluorouracil	
Val → Ala	III	1
Thr → ,,	I	1
Ser → Gly	IV	1
	Hydroxylamine	
Pro → Leu	I	1

with a diagram depicting the triplet conversions theoretically possible by nitrous acid treatment, it becomes clear that the genetic code is degenerate[14].

The high frequency with which certain amino acid exchanges occur is striking (Table 2). For example, threonine has been replaced a total of twelve times in different peptides and mutants but by either isoleucine or methionine only. Thus, there are restrictions in the amino acid exchanges produced by nitrous acid treatment of TMV-RNA. These restrictions as contrasted to the significantly larger number of amino acid exchanges theoretically possible by nitrous acid treatment might be explained as follows: (*a*) Only a few of the amino acids are coded by more than one triplet, i.e. the degree of degeneracy of the genetic code is low. (*b*) In the case of a highly degenerate code those triplets which determine the same amino acid do not represent a random selection from the 64 possible triplets[14]. According to the results obtained from cell-free system studies[15,16] the second possibility is much more probable than the first.

In Fig. 1 are listed those mutants in which the exact positions of the amino acid exchanges have been localized. Only those positions are listed which differ from the parent strain. The maximum number of exchanges which occur in any of the chemically induced mutants is two. The fact that there is more than one amino acid replacement in the coat protein can be explained on the basis that in many experiments with a large number of deaminations per TMV-RNA more than one nucleotide within the cistron for the coat protein was changed[14]. The finding that for all chemically induced and spontaneous mutants two neighbouring amino acids were never exchanged is a good indication that the code is non-overlapping. Only under very special assumptions[17] are other types of codes compatible with these results.

As mentioned above, one of the two mutagenic alterations caused by nitrous acid treatment is the conversion of cytosine to uracil. The triplet CCC should be converted stepwise to the triplet UUU by nitrous acid treatment (Fig. 2). According to the results obtained from cell-free system studies, proline is coded by C-rich triplets and phenylalanine by U-rich triplets. Two conclusions may be drawn from Fig. 2: (1) The infecting RNA strand, or an RNA strand which is identical to it in nucleotide sequence and not complementary, serves as template for biosynthesis of the TMV coat protein. Only in this case are the observed amino acid exchanges explained. No amino acid exchanges which lead to proline (CCC or CUC) or which are directed away from phenylalanine (UUU or UCU) should occur in nitrous acid mutants. This expectation was confirmed experimentally, as such amino acid exchanges have never been found among the numerous exchanges observed with the nitrous acid mutants. Such changes occur, however, among the far smaller number of spontaneous mutants studied. (2) According to Fig. 2 the nucleotide

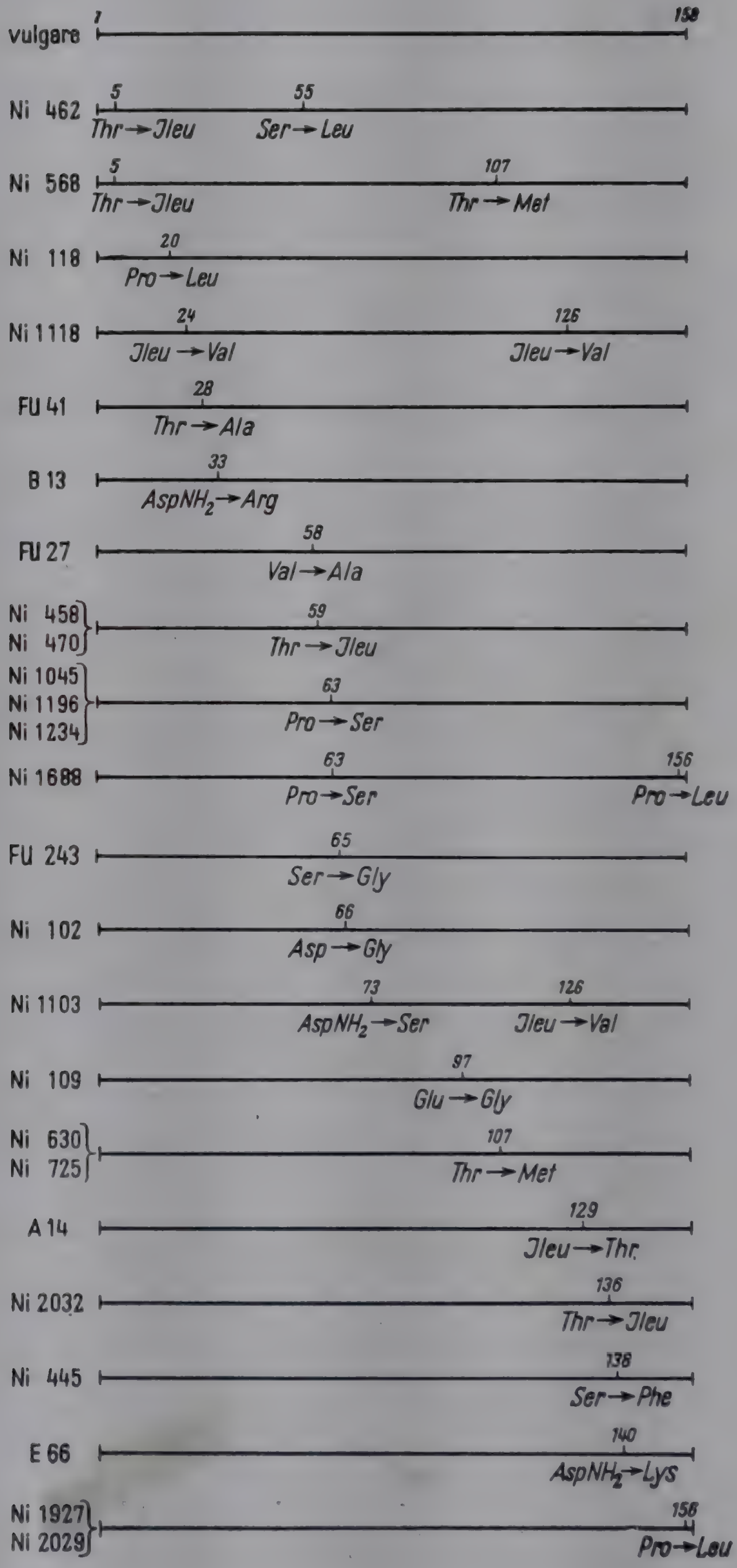
vulgare 1 158
Ni 462 5 Thr→Jleu 55 Ser→Leu
Ni 568 5 Thr→Jleu 107 Thr→Met
Ni 118 20 Pro→Leu
Ni 1118 24 Jleu→Val 126 Jleu→Val
FU 41 28 Thr→Ala
B 13 33 AspNH2→Arg
FU 27 58 Val→Ala
Ni 458 Ni 470 59 Thr→Jleu
Ni 1045 Ni 1196 Ni 1234 63 Pro→Ser
Ni 1688 63 Pro→Ser 156 Pro→Leu
FU 243 65 Ser→Gly
Ni 102 66 Asp→Gly
Ni 1103 73 AspNH2→Ser 126 Jleu→Val
Ni 109 97 Glu→Gly
Ni 630 Ni 725 107 Thr→Met
A 14 129 Jleu→Thr
Ni 2032 136 Thr→Jleu
Ni 445 138 Ser→Phe
E 66 140 AspNH2→Lys
Ni 1927 Ni 2029 156 Pro→Leu

FIG. 1—LOCATION OF AMINO ACID EXCHANGES IN MUTANTS OF TMV

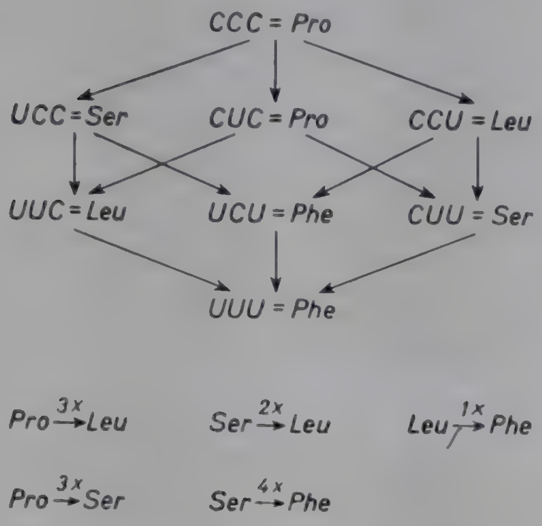

FIG. 2—DIAGRAM SHOWING THE NITROUS ACID INDUCED BASE CONVERSIONS LEADING FROM THE TRIPLET CCC TO UUU: Amino acid exchanges found in nitrous acid induced mutants are combined with this diagram

sequences for the triplets which code proline are CUC in addition to CCC, and for phenylalanine UCU besides UUU. By combining the results from amino acid exchange studies with the results obtained from cell-free system studies, one can try to determine the nucleotide sequences for the codons of a number of amino acids[18,19].

So far we have mentioned only mutants of TMV which were chemically induced or which occurred spontaneously in the greenhouse. They are often indistinguishable in protein moiety from the parent strain or differ by only one or two amino acid exchanges. We have, in addition, determined the amino acid sequence of TMV strains which were isolated in nature and which are only slightly related to the TMV wild strain vulgare. Two of these are dahlemense, a TMV strain isolated from tomatoes by Melchers[20] and the TMV strain M, a strain with mild symptoms, which we obtained from Dr W. H. Takahashi, Department of Plant Pathology, Berkeley. From amino acid sequence analysis of both strains[12] (Wittmann and Rentschler, to be published) it was found that the amino acid sequence of dahlemense differs from that of vulgare in 29 positions. The number of amino acid differences between strain M and vulgare is about 35. In the three strains the amino acids in 70% of the amino acid positions are identical. In each case the total number of amino acids is the same, namely 158. It has been shown by X-ray diffraction studies[22] that differences in structure exist between vulgare and dahlemense: in vulgare the pitch of the helix is always 23 A., whereas with dahlemense it is alternatingly 5% larger or smaller.

Unfortunately, little is known about the tertiary structure of the protein subunits, because it has not yet been possible to obtain crystals of TMV of sufficient size and in sufficient quantity for X-ray diffraction studies. Thus, one is dependent upon indirect methods[19,23–30]. We have made

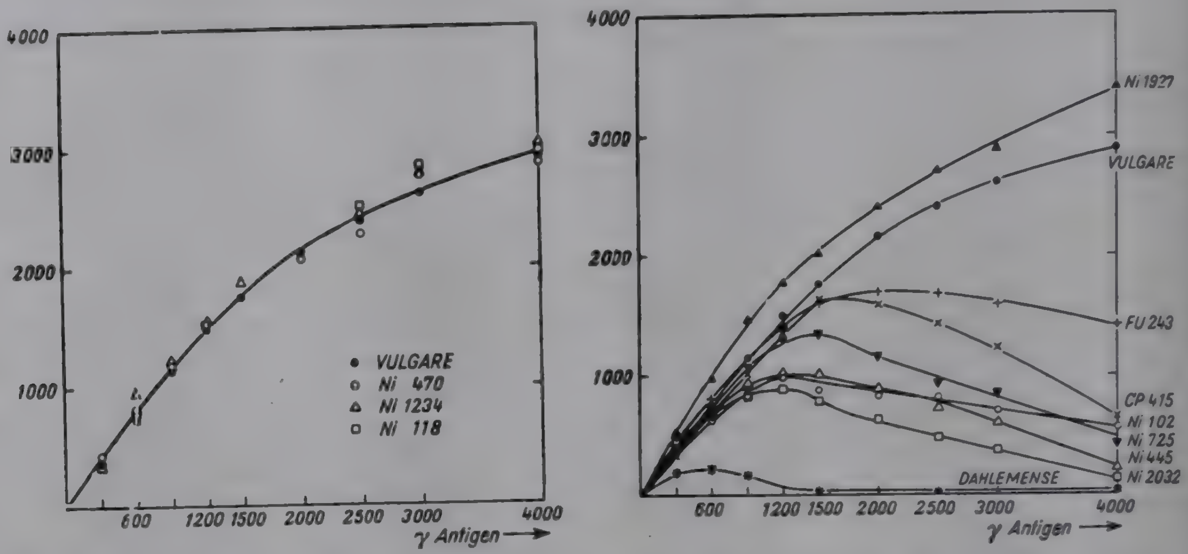

FIG. 3—PRECIPITATION OF SOME TMV MUTANTS WITH ANTISERUM PREPARED AGAINST VULGARE: Ordinate: γ precipitate

use of two of these methods: free boundary electrophoresis and serology. Conclusions regarding the fine structure of the virus subunit may be drawn by combining information about the exact amino acid sequence of TMV mutants and strains and differences in their electrophoretic and serological properties.

Fig. 3 shows the serological reaction of various mutants with antiserum against the wild strain vulgare[31]. On the left half of Fig. 3 are presented those strains which are serologically indistinguishable from vulgare, although each differs in amino acid sequence from vulgare. On the right half of Fig. 3 are shown mutants which differ in their serological reaction from vulgare. Most of these give a weaker precipitation reaction with antiserum against vulgare than vulgare itself. As expected, one of the above mentioned TMV strains, dahlemense, which differs in amino acid sequence from vulgare in 29 positions, exhibits an extremely weak precipitation reaction with vulgare antiserum.

It was surprising to discover that one of the mutants investigated, Ni 1927, reacted to a distinctly larger degree with vulgare antiserum than vulgare itself. In this mutant, Ni 1927, leucine occurs at position 156. Proline is found at the corresponding position in vulgare. A mutant with the same replacement (proline→leucine) has been found by Tsugita and Fraenkel-Conrat[1]. If vulgare is treated with carboxypeptidase A, then only one amino acid is split off, namely the C-terminal threonine in the last position 158, since the further action of carboxypeptidase is hindered by proline[32]. However, in the case of the mutant, carboxypeptidase action is no longer blocked by proline, and the enzyme cleaves further amino acids in addition to the last. If the intact virus Ni 1927 is treated with carboxypeptidase, then the last three amino acids (threonine, alanine and leucine) are cleaved from all of the virus subunits, with no loss of the fourth from the last position (glycine). If one first denatures the virus protein, then glycine is cleaved by the enzyme and cleavage continues far beyond this position; i.e. the native protein structure is requisite to the limitation of carboxypeptidase action on Ni 1927 to the last three amino acids.

We have prepared antisera against the following antigens: (1) Untreated vulgare; (2) vulgare treated with carboxypeptidase (vulgare+C'ase); (3) Untreated Ni 1927; (4) Ni 1927 treated with carboxypeptidase (Ni 1927+C'ase). The reaction of each of the four antisera with each of the four antigens was examined by the precipitation test (Figs. 4 and 5) and the exhaustion test[30]. As indicated in the figures, Ni 1927 +C'ase antigen gives the best reaction as compared with the other antigens, independent of which antiserum is utilized. As mentioned above, Ni 1927

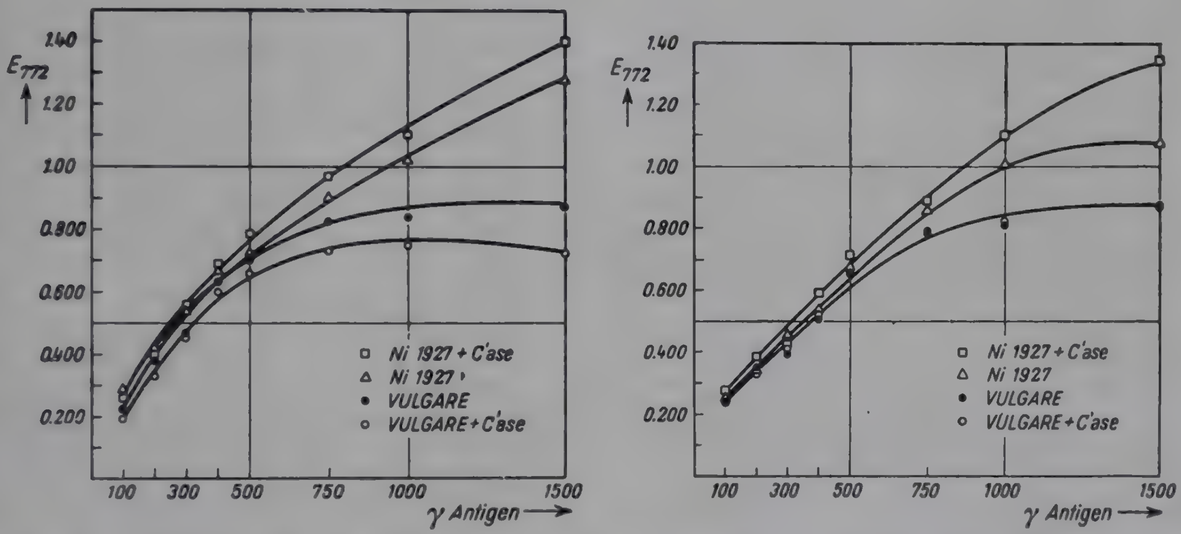

FIG. 4—PRECIPITATION OF FOUR ANTIGENS (see text) WITH ANTISERUM PREPARED AGAINST VULGARE (LEFT) AND VULGARE TREATED WITH CARBOXYPEPTIDASE (RIGHT)

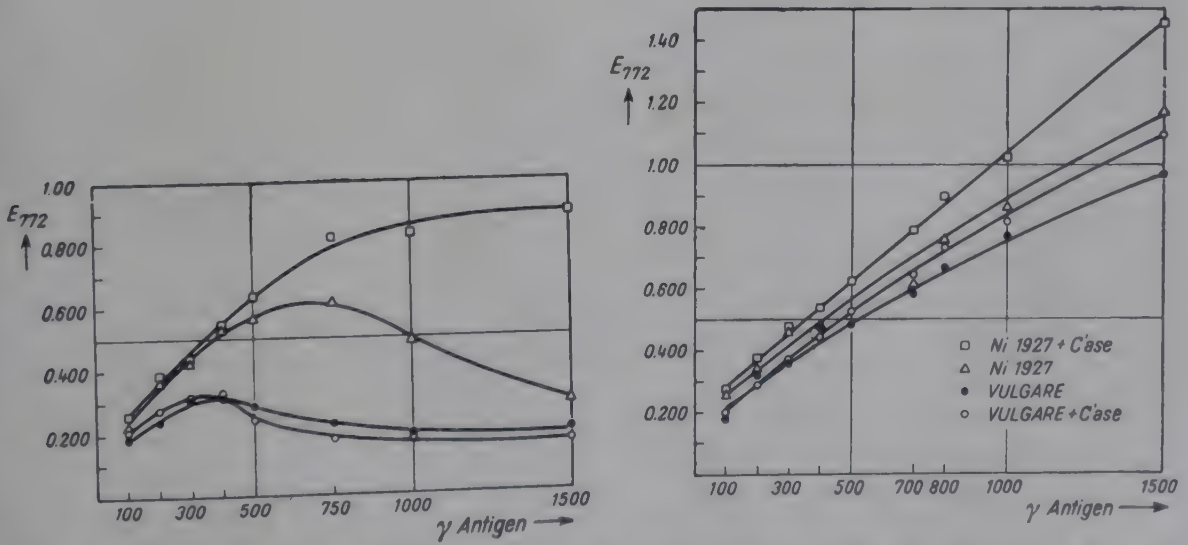

FIG. 5—PRECIPITATION OF FOUR ANTIGENS (see text) WITH ANTISERUM PREPARED AGAINST THE TMV MUTANT Ni 1927 (LEFT) AND Ni 1927 TREATED WITH CARBOXYPEPTIDASE (RIGHT)

gives more complete precipitation with vulgare antiserum than vulgare itself (Fig. 3).

A probable explanation for this finding is as follows: the last three amino acids (pos. 156-158) of the TMV protein chain projects as a free end from the virus subunit so that they are susceptible to cleavage by carboxypeptidase. The binding between the antibody and the viral surface increases, the less this reaction is sterically hindered by the projecting end of the protein chain. There is a greater hindrance effect with vulgare than with Ni 1927, since the projecting end of the protein chain of vulgare, which contains proline, is more rigid than that of Ni 1927 in which proline is replaced with leucine. In the case of Ni 1927, this end is more flexible and can assume a position which interferes with binding of the antibody to the virus to a lesser degree.

Ultracentrifuge studies show that cleavage of the last three amino acids of the protein chain has no observable influence on the aggregation of A protein particles, each of which contains three virus protein subunits, to virus rods. On the other hand, a quite distinct difference between Ni 1927 and Ni 1927+C'ase has been observed in electrophoretic studies. The electrophoretic mobility of Ni 1927+C'ase is greater than that of Ni 1927 both under acid conditions (*p*H 2) in which only the basic groups are charged and under alkaline conditions (*p*H 8) in which the difference in charge between the acidic and basic groups is effective for the electrophoretic mobility[30]. These results together with results of earlier electrophoretic studies[27] lead to conclusions relating to questions of the fine structure of the virus unit.

SUMMARY

Mutants of TMV were produced by treatment with specific mutagenic agents (nitrous acid, hydroxylamine, 5-fluorouracil). Investigations were carried out on the proteins from a total of 187 chemically induced and spontaneous mutants; they differ from the parent strain by zero, one or two amino acids. On the other hand, the number of amino acid differences was found to be considerably larger between TMV strains which were isolated in nature and whose exact amino acid sequences were determined. A comparison of the sequences of three TMV strains shows that the amino acids in only 70% of the amino acid positions are identical.

From these investigations conclusions may be drawn concerning properties of the genetic code including overlapping, degeneracy, size of codon, nucleotide sequence within the codon, and the question of whether the plus RNA strand or its complementary strand serves as template for the synthesis of TMV protein.

Information about the fine-structure of the virus was obtained from serological and physico-chemical investigations of TMV mutants whose exact amino acid sequences are known.

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RNA-induced Synthesis of Organ Specific Proteins*

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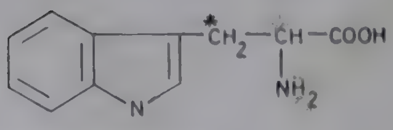
Previous experiments have shown that ascites tumor cells can be induced by RNA isolated from the mouse, the rat or the calf liver to synthesize protein (enzyme) characteristic of the liver[1-8]. This RNA is heterogeneous. The fraction that is responsible for the activity has not been clearly defined. Recent study on the liver RNA-induced biosynthesis of serum albumin has presented evidence for the nucleolar II fraction (messenger RNA)[7]. However, distribution studies of the exogenous RNA in the treated cells and its subsequent effect indicated that there is an additional role, namely, to activate differentially the gene function[4]. As a result, the activated genome would synthesize new messenger RNA, which in turn acts as template for the synthesis of specific proteins.

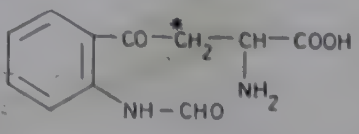
What I plan to discuss is to briefly review the data concerning the acquired protein biosynthesis in mammalian cells. In the brief time available, I would like to discuss three RNA-induced proteins, namely, tryptophane pyrrolase, serum albumin and hemoglobin.

TRYPTOPHANE PYRROLASE

Tryptophane pyrrolase (T.P.) is an enzyme found in the liver of vertebrates. It is inducible by the substrate, tryptophane, as well as cortisone[9]. Activity of the enzyme is estimated by its capacity to release kynurenine (K) when incubated with tryptophane. Two steps are involved in the conversion of tryptophane into kynurenine, namely,

$$(1)\quad \text{Tryptophane} \xrightarrow[O_2 \text{ and } H_2O]{\text{T. P.}} \text{Formyl Kynurenine}$$





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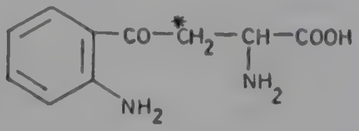
(2) $\text{Formyl kynurenine} \xrightarrow[H_2O]{\text{Formylase}} \text{Kynurenine} + HCOOH$



The enzyme formylase in the second step is not inducible, but is present in livers of all animals with approximately the same high content. In an inducible system of T. P. activity, the release of K involves the participation of formylase, whose presence may either be an integral part of the cells or acquired through metabolic manipulation. If the liver-RNA is indeed capable of inducing serum albumin, catalase, glucose-6-phosphatase, arginase and T. P. activity in mammalian cells, it is reasonable to assume that formylase may also be induced by the RNA. However, no analysis of formylase activity has yet been made.

T. P. activity in the liver-RNA treated ascites cells was examined by the procedure of Knox[9]. The amount of K released was estimated by the ultraviolet absorption spectrum between 310 and 380 mμ. K was characterized by higher absorption at 365 mμ than at 320 mμ. Typical spectrum of Kynurenine sulphate in the presence of ascorbic acid (0·03 *M* activater of T. P.) is shown by Fig. 1. It can be seen that the amount less than 4 μg. does not yield spectrum of K. 4 μg. and above provides typical K spectra. In view of the small amount (expected) of K produced in an induced system, we decided to analyse it by two procedures: (1) the standard procedure of Knox and (2) the isotopic method using ^{14}C-tryptophane as a substrate[10]. ^{14}C-Kynurenine was isolated by paper chromatography. Its radioactivity was counted by the Nuclear Chicago gas flow autoscaler.

Fig. 2 illustrates the ultraviolet absorption spectra of K in control (C_2) and the liver RNA treated ascites cells ($E_{1,2}$). Absorption spectrum of $E_{1,2}$ indicates the presence of K. When 5 μg. K-sulphate was added to both C_2 and $E_{1,2}$, the absorption spectrum showed the presence of K in E, but not in C (compare with Fig. 1).

Analyses of T. P. activity in the mouse ascites cells revealed that approximately 50% of our RNA-treated mice (treatment involves intraperitoneal injection of liver RNA into mice carrying ascites tumor) gave positive result. The reason for the absence of activity in other experiments was not clear at the present time. It might be possible that during the generation time of the tumor cells one phase may be more reactive to RNA treatment than the other.

The approach using ^{14}C-tryptophane was proceeded in the same way as the above, except that an aliquot was used for chromatographic isolation of ^{14}C-K. Two-dimensional paper chromatography Whatman

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*Denotes the metabolic fate of the atom in the tryptophane molecule.

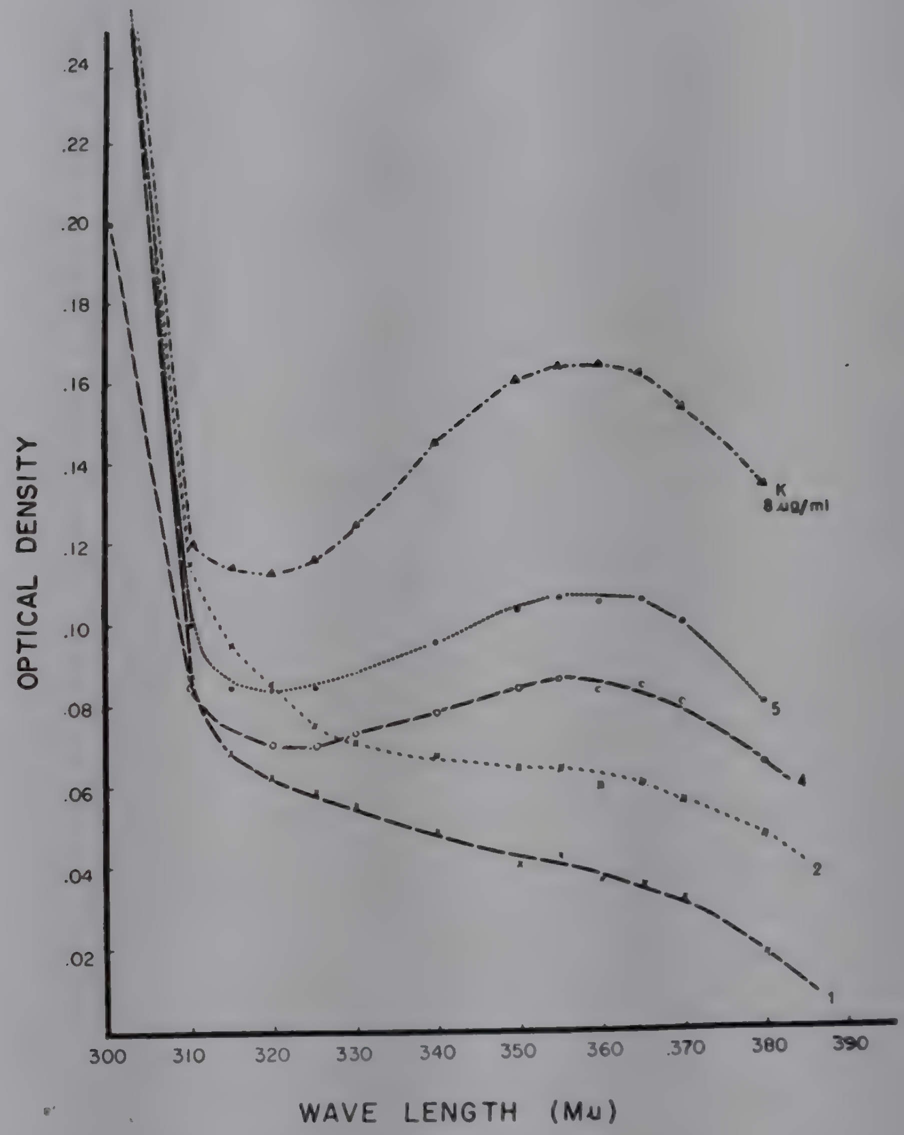

Fig. 1—Ultraviolet absorption spectra of kynurenine sulphate in ascorbic acid solution (0·03M): 4μg. and above gives spectra characteristic of kynurenine; below 4μg. it cannot be detected by this procedure

Paper No. 1) was done with solvents (i) *n*-butanol : acetic acid : water (3:1:1), and (ii) water. K is fluorescent and can easily be spotted by ultraviolet lamp. Cold K (5 μg./spot) was added as a carrier to the sample to be chromatographed. Elution was done with water. Both the absorption spectrum and the radioactivity counting were recorded. The radioactivity ratio between E and C was 2·7. It was assumed that the high C activity might be due to trace contamination of ^{14}C-tryptophane. Rechromatography of the eluted material has been done[10]. Counts of radioactivity were 3600 counts in E and 1000 counts in C (a ratio of 3·6), thus validating our assumption.

Serum Albumin

Serum albumin (S.A.) is produced by the liver cells. Neither the mouse ascites tumor nor any other somatic cells are capable of synthesizing it.

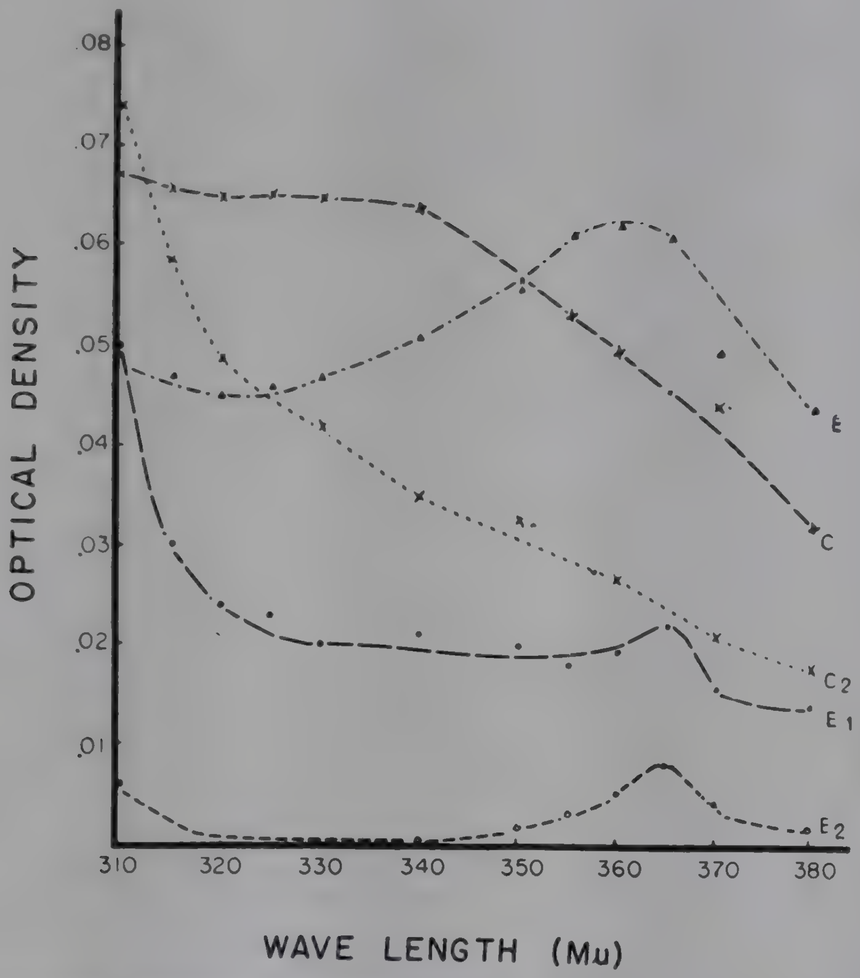

FIG. 2—ULTRAVIOLET ABSORPTION SPECTRA OF THE SUPERNATANT OF THE CONTROL (C_2–C) AND THE EXPERIMENTAL CELLULAR HOMOGENATES ($E_{1,2}$–E): These homogenates were incubated with tryptophane at 37°C. for an hour and immediately deproteinized (Compare with Fig. 1 and see text)

Following the treatment with liver RNA, the mouse ascites cells acquired the capacity to synthesize S.A. Procedure for the isolation and identification of S.A. has already been reported. Fig. 3 illustrates the proteins synthesized and their rate of synthesis. It is noted that the rate of general (GP) and lipoprotein (LP) synthesis remains almost steady, while that of S. A. is directly proportional to the amount of RNA used to treat the cells[2]. Two possibilities can account for the appearance of S.A. in the treated cells: (1) RNA used is contaminated with S. A. and/or (2) S. A synthesis is induced by RNA. The former is unlikely because (*a*) antigenic material cannot be demonstrated by treating the RNA preparation with the rabbit antiserum against bovine or rat serum albumin[2], and (*b*) biosynthesis of S. A. in the treated cells is linear with time (Fig. 4).

The RNA-induced synthesis of S. A. has lately been confirmed and extended by Zimmermann *et al.*[7] These authors prepared different fractions of RNA from the rat and calf livers and found that nucleolar II fraction (messenger RNA) is the material for *in vitro* induction of S. A. biosynthesis. It may be relevant to mention at this point thatc ytoplasmic messenger

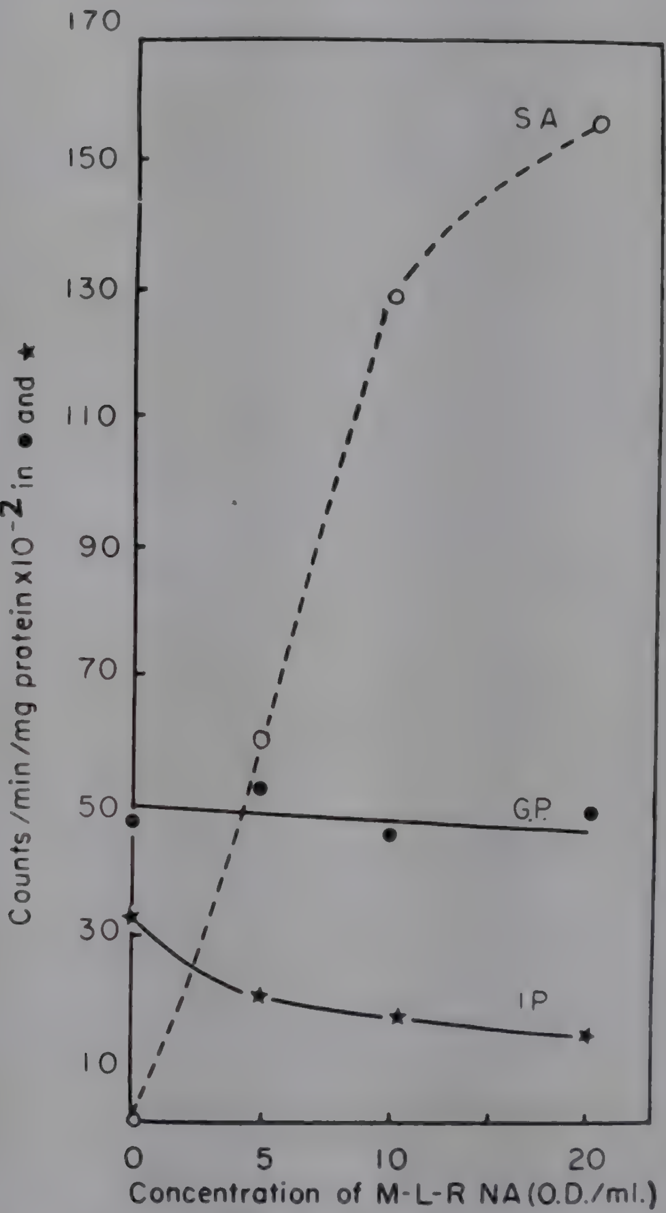

FIG. 3—EFFECT OF MOUSE LIVER RNA ON THE INCORPORATION OF DL-LEUCINE-^{14}C INTO PROTEIN IN THE LIVER RNA TREATED ASCITES CELLS IN 2·5 HR: S.A., Serum albumin; G. P., general protein and L. P., lipoprotein. The control (untreated) cells have practically no S.A. produced

RNA can readily be isolated and has been proved reasonably stable[11]. We believe that the RNA we used to induce S. A. synthesis contains this fraction (see our study using actinomycin D[4]).

HEMOGLOBIN

The third protein that I would like to say few words about is hemoglobin. Thanks to Dr Borsook of the California Institute of Technology, rabbit reticulocytes have been the favourite material for *in vitro* synthesis of hemoglobin. The major problem of the latter is, how to identify the new hemoglobin molecules ? The procedure developed by Ingram[12] to

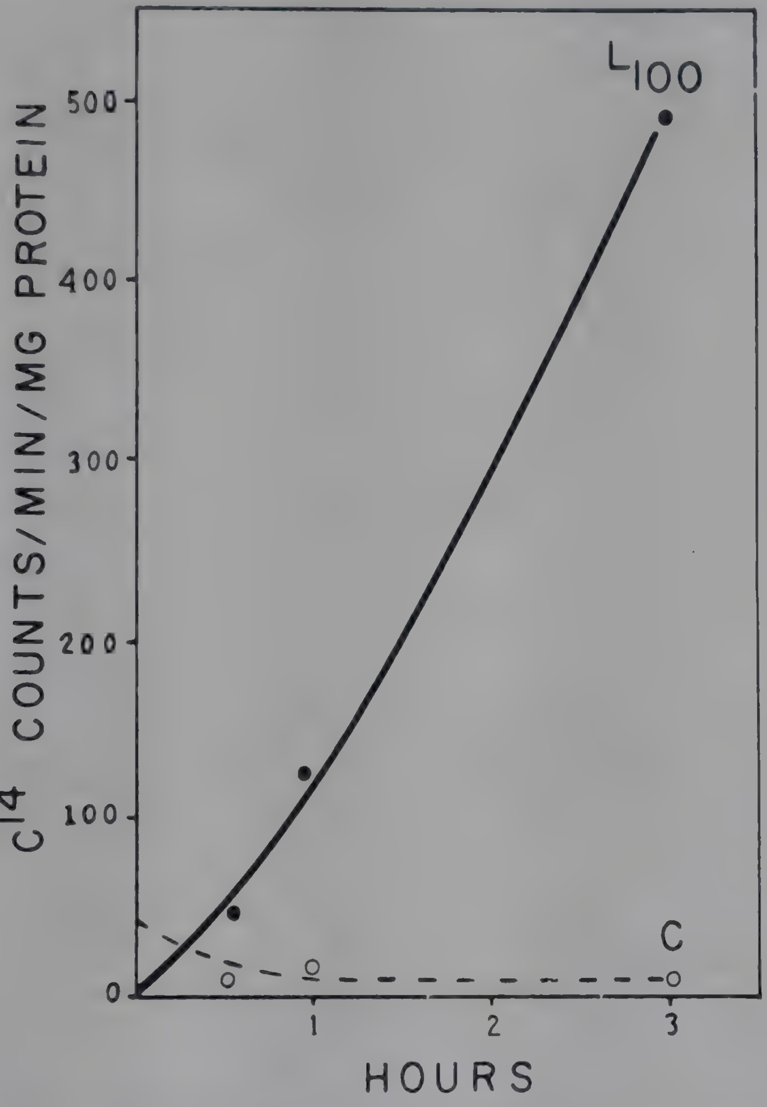

FIG. 4—TIME COURSE OF THE INDUCED BIOSYNTHESIS OF S.A. BY THE LIVER RNA TREATED EHRLICH ASCITES CELLS: The newly synthesized S.A. is expressed by the counts of ^{14}C activity. L_{100} indicates the optical density at 260 mμ of liver RNA (OD 100/ml.=5 mg./ml.)

differentiate normal human hemoglobin from that of sickle-cell anemic patient has been adopted by most investigators.

Human bone marrow cells were treated with ribonucleoprotein from the counterpart of the sickle-cell anemic patient or vice versa and then incubated at 37°C. for 24 hr. The hemoglobins isolated from these cells and those without treatment were subjected to electrophoresis and column chromatography. The result of these analyses showed the presence of an abnormal hemoglobin component[13]. Finger-printing of the abnormal component revealed the presence of additional peptides.

Rabbit reticulocytes were incubated at 37°C. with the RNA prepared from duck erythrocytes or vice versa[14]. IRC-50 column chromatography of the hemoglobins obtained from these cells showed the existence of a component of duck hemoglobin. Conversely, the RNA-treated duck cells had a component of the rabbit hemoglobin. Finger-printing of either of

TABLE 1—Precipitation Tests of Hemoglobins

Antigen / Antibody	Rabbit Hb 1%-0·2ml.	Chicken RNA-treated Rabbit Hb 1%-0·2ml.	Chicken Hb 1%-0·2ml.
Rabbit antiserum against chicken Hb	170 μg.	360 μg.	1130 μg.
Antibody / Antigen	The supernatant above	The supernatant above	The supernatant above
Chicken Hb 1%-0·2ml.	136 μg.	70 μg.	43 μg.

TABLE 2—Antigenic Activity of Hemoglobins

Antigen / Antibody	Rabbit Hb	Chicken RNA-treated Rabbit Hb	Chicken Hb
Rabbit Hb absorbed Rabbit antiserum against Chicken Hb	trace	28 μg.	85 μg.

these hemoglobins revealed extra peptides in addition to those found in the control (untreated) cells. It appears, therefore, that RNA treatment has initiated the synthesis of new hemoglobin molecules in the alien hemoglobin-producing cells.

Our own approach to the RNA-induced hemoglobin production differs from the above in two points: (1) chicken blood RNA was used to treat rabbit reticulocytes *in vivo*. This was accomplished by a series of RNA injections into the phenylhydrazine produced anemic rabbits, and (2) hemoglobin of the treated and control rabbits were analysed by immunological tests and CMC column chromatography.

Rabbit antiserum against chicken hemoglobin was produced in our laboratory by a series of intravenous injections of the purified chicken hemoglobin. Table 1 gives the result of precipitation tests. It can be seen that precipitates obtained from the RNA-treated rabbit hemoglobin are in between those of the rabbit and the chicken[15].

The rabbit antiserum was absorbed with rabbit hemoglobin. It was found that the absorbed antiserum reacted to the RNA-treated rabbit hemoglobin in between those of the rabbit and chicken hemoglobins (Table 2).

Preliminary study with CMC column chromatography, electropheresis and finger-printing technique revealed the presence of chicken hemoglobin component in the chicken RNA treated rabbit hemoglobin (unpublished).

SUMMARY

RNAs isolated from calf liver and chicken erythrocytes were used for the induction of organ specific proteins. Liver RNA induces mouse ascites cells to synthesize tryptophane pyrrolase and serum albumin. Chicken erythrocytes RNA causes rabbit reticulocytes to produce hemoglobin containing a component of the chicken hemoglobin. Therefore, the RNA used in these experiments carries the genetic code of the RNA tissue source.

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The Translation of Genetic Information into Proteins on Polyribosomes

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DNA is the primary storehouse of genetic information in the cell. We believe that the genetic information is encoded in the sequence of nucleotides which are found in the polynucleotide chains. DNA itself is a two-stranded helical molecule built out of complementary halves; this makes possible the self replication which is implicit in cell division. In recent years, we have come to learn a great deal about the mechanism for reading out the information which is found in DNA. We now know that it is a two step process involving the formation of a single stranded ribonucleotide polymer of messenger RNA which has the property of directing the synthesis of protein. The formation of messenger RNA from DNA is called "transcription", while the formation of polypeptide chains from messenger RNA is called "translation". Here we shall discuss various aspects of the translation mechanism, emphasizing in part, properties of the system which have been well established experimentally, but we shall also consider aspects of the system which have not been worked upon as yet.

The first indication of the existence of messenger RNA came from work with bacteria in which it was possible to demonstrate the existence of an RNA fraction that is manufactured very rapidly on DNA and released to direct the synthesis of specific proteins[1-3]. Before that time, it was generally felt that RNA was active in directing the synthesis of protein, but the structural RNA of the ribosome had not been differentiated from the messenger or informational RNA. The latter has a sequence of nucleotides which contains information that is translated into a sequence of amino acids to form polypeptide chains. A short time later Nirenberg and Matthaei[4] demonstrated a rather striking action of the synthetic polynucleotide, polyuridylic acid (poly U), in stimulating the formation of polyphenylalanine, clearly demonstrating the messenger role of this polynucleotide. An important consideration in understanding this system is the determination of the "coding ratio", the number of nucleotides in messenger RNA which are required to specify an individual amino acid. Genetic experiments by Crick and his collaborators[5] strongly suggested that the coding ratio was three. This provides a quantitative basis for relating the amount of messenger RNA which is necessary to code for a polypeptide of known length.

For many years, it has been known that the assembly of polypeptide chains takes place on ribosomes, small particles which are roughly spherical in the electron microscope with a diameter in the neighbourhood of 200A. These are built out of RNA and protein. Amino acids are carried to the ribosomes attached to soluble RNA molecules. During protein synthesis, polypeptide chains are assembled on ribosomes starting from the N-terminal amino acid and then sequentially working towards the C-terminal end. This was first clearly demonstrated in experiments with hemoglobin synthesis[6,7]. One would expect that there is a relation between the length of messenger RNA needed to code for a given polypeptide chain and the number of amino acids which are found in that polypeptide chain. This relationship was also first clearly seen in work on rabbit reticulocytes which are largely manufacturing the single protein hemoglobin[8-10]. Hemoglobin is composed of polypeptide chains which contain approximately 150 amino acids. A coding ratio of three nucleotides for each amino acid implies a polynucleotide chain in the vicinity of 450 nucleotides. Since the stable conformation of a polynucleotide chain is one in which the planar purine and pyrimidine residues are stacked upon each other with a spacing of 3·4 A. per nucleotide, we can estimate that the messenger strand for the hemoglobin polypeptide chain is approximately 1500 A.

The reticulocyte experiments involve a brief incubation of intact cells with ^{14}C-labelled amino acids. The cells are lysed and sedimented on a sucrose gradient. Analysis of the gradient reveals that the nascent or growing polypeptide chains are associated with clusters of ribosomes called polyribosomes or polysomes which sediment more rapidly than the single ribosomes as shown in Fig. 1[10]. For hemoglobin synthesis, the predominant species is a pentaribosomal unit; however, there are substantial numbers of tetra- and hexaribosomal units as shown by the dotted lines in Fig. 1. These distributions were determined by studying these fractions in the electron microscope. Fig. 2A shows a platinum shadowed electron micrograph from the centre of the polysomal distribution. It can be seen that the clusters frequently have a rosette configuration but occasionally are found in an extended form. These ribosomes are held together by RNA since it has been shown that very low concentrations of the enzyme ribonuclease acting at 2°C. convert these into single ribosomes which still have the nascent radioactive chains attached to them. The RNA strands connecting the ribosomes can be seen in positively stained electron microscopy as shown in Fig. 2B[11]. Here reticulocyte polysomes are stained with

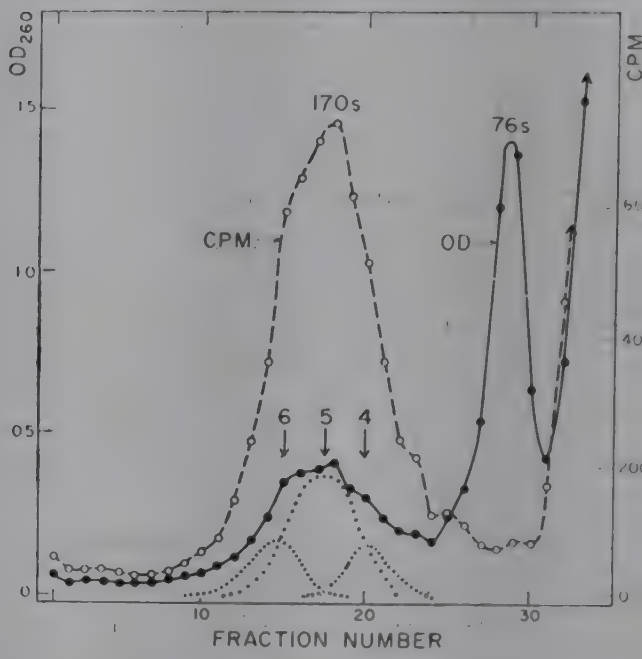


Fig. 1—Sucrose density gradient analysis of lysed rabbit reticulocytes: The cells had been incubated for 45 seconds with ^{14}C-labelled amino acids before lysis. The solid line represents the optical density at 260 mμ while the dashed line represents the radioactivity. The direction of sedimentation is to the left. The solid line shows a peak labelled 76s which represents single ribosomes while the broader peak sedimenting more rapidly represents the polyribosomal distribution. The samples from the polyribosomal peak were examined in the electron microscope and the dotted distributions labelled 4, 5 and 6 represent the size of the polysomal clusters. The pentamer ribosomal unit is the most predominant but there are substantial numbers of hexamers and tetramers of ribosomes as well[10]

uranyl acetate and a thin 10-15A. wide filament can be seen connecting the ribosomes. This diameter is consistent with that of a single strand of RNA. The distance between the ribosomes is variable ranging from 50 to 150A. Since the width of the ribosomes is approximately 220A., the total contour length of the reticulocyte polyribosome is very close to 1500A., which has been estimated as the length of the messenger required to code for hemoglobin polypeptide chains[8].

Polyribosome Mechanism in Protein Synthesis

If the messenger RNA strand contains sequence information for the stepwise addition for amino acids to a polypeptide chain, it is reasonable to assume that the ribosome reads this message by moving along the RNA strand, sequentially adding the individual amino acids[12]. This mechanism is illustrated in Fig. 3[13], in which a shaded ribosome is about to attach to one end of the messenger strand. After attachment to the messenger strand, the assembly of the polypeptide chain is initiated and as the ribosome moves to the right the polypeptide chain is elongated. When the ribosome reaches the end of the messenger strand, it is detached and the polypeptide strand is released. A variety of experiments have been carried out to test various parts of this polyribosomal mechanism and they will be mentioned here briefly.

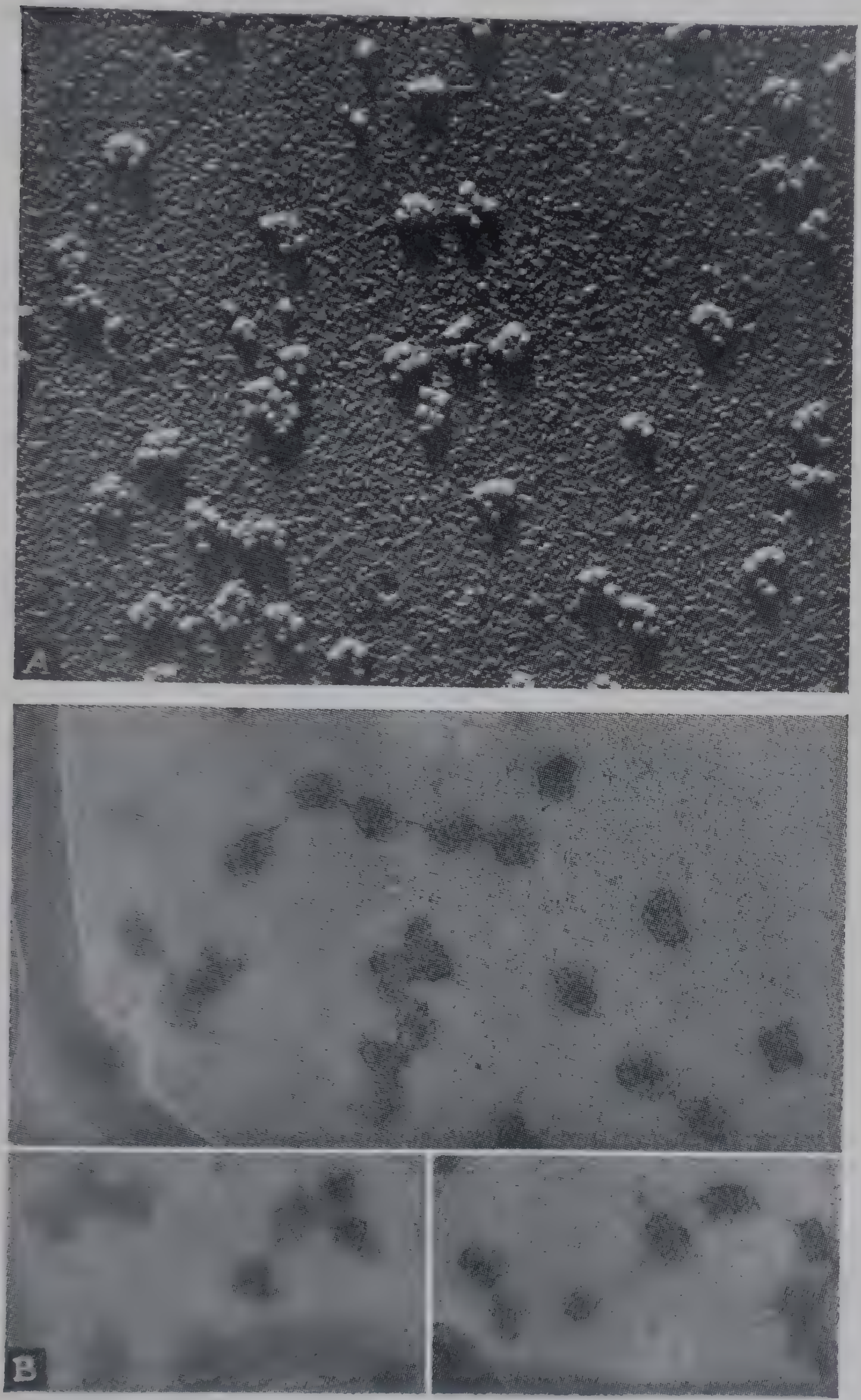


Fig. 2—Electron micrographs of reticulocyte polyribosomes: (A) The polyribosomes have been isolated and shadowed with platinum at a 5:1 angle. They appear characteristically as clusters, in some cases closely bunched together and at other times in a more or less extended array. The inter-ribosomal gap varies from 50A to 150A. (Ref. 10). (B) Reticulocyte polyribosomes stained with uranyl acetate and examined at higher magnification[11]. Here each ribosome can be seen as a somewhat irregular shaped object with a diameter of approximately 220A. Extending between the ribosomes can be seen a very thin, positively stained thread with a diameter of 10-15A. This thread, presumably messenger RNA, is seen to pass through the ribosomes; occasionally it goes through somewhat eccentrically so that it is possible that the messenger is lying between the 50s and 30s subunits of the ribosome

1. The mechanism explains the distribution of polyribosome size seen in the reticulocyte which is manufacturing only one protein, hemoglobin. In the electron microscope, the spacing between ribosomes on the polyribosomal structure is variable, implying that the movement of the ribosomes is statistical rather than cooperative. This means that in some instances, a ribosome will detach at the end of the messenger strand before a new one has attached, giving rise to a transient population of tetramers. Alternatively, attachment at the other end before detachment occurs will give rise to a transient population of hexamers. This explains the naturally occurring distribution which is seen in reticulocytes which are synthesizing a single protein as shown in Fig. 1[10].

2. Fig. 3 implies that single ribosomes are not active in protein synthesis because they lack messenger RNA and not because of any intrinsic defect in the ribosome. The ability of the single ribosomes to be active in protein synthesis has been demonstrated in the reticulocyte systems by the addition of the synthetic messenger, poly U[9,10]. If enough poly U molecules are added to a lysate so that each ribosome has its own poly U messenger strand, then poly U polyribosomes are not formed. Under these conditions, single ribosomes incorporate phenylalanine actively while the polysomes are still manufacturing hemoglobin[14].

3. Fig. 3 implies that there is a single growing polypeptide chain attached to each ribosome in the polysome. This has been determined by quantitative measurements in reticulocytes[15]. By using the known sequence of amino acids in the growing hemoglobin polypeptide chains, it is possible to measure the number of growing chains by measuring the incorporation

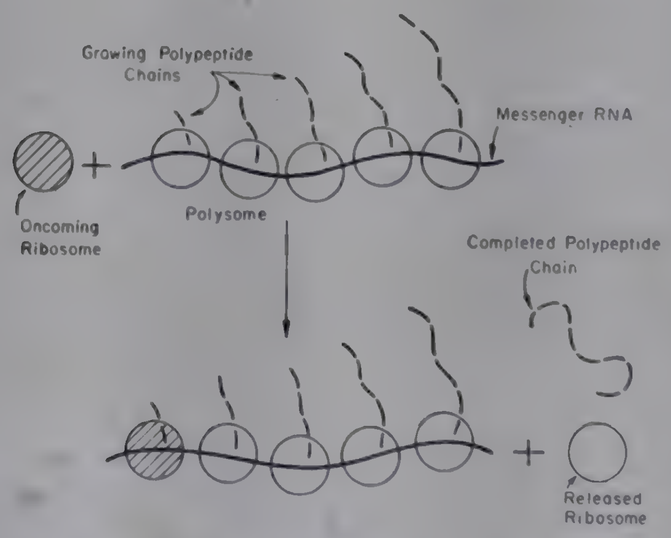


FIG. 3—A SCHEMATIC MODEL OF POLYSOME FUNCTION[13]: The solid line represents the messenger RNA along which the ribosomes are moving. Accompanying the movement of the ribosomes is an elongation of the polypeptide chain as shown by the dashed line. Recent experiments have shown that the growing polypeptide chains are, in fact, coiled so that they have native secondary and tertiary structure while they are still attached to the polyribosomes[35]

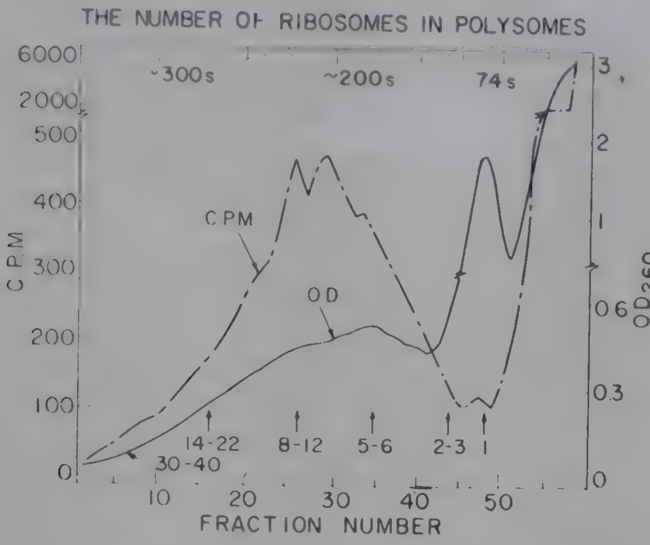


FIG. 4—A SUCROSE DENSITY GRADIENT ANALYSIS OF HELA CELL POLYSOMES LABELLED WITH A SHORT PULSE OF ^{14}C AMINO ACIDS: The solid line represents the optical density and the dashed line the radioactivity. The sharp peak at 74s represents the sedimentation of single ribosomes. It can be seen that most of the radioactivity is located in the polyribosomal peak rather than with the single ribosomes. The numbers along the bottom refer to the size of the polyribosomal clusters which are seen in an electron microscope analysis of various fractions[16]

of radioactive leucine on to active polyribosomes. These experiments led to the conclusion that there is one growing polypeptide chain per ribosome in the polysomal fraction.

4. The reticulocyte is a specialized cell which is manufacturing largely a single protein. However, in the mammalian HeLa cell grown in tissue culture, a large variety of proteins are being made. This is reflected in the distribution of polysome size which is seen in the cytoplasmic extract as shown in Fig. 4. The cells were exposed for a short time period to ^{14}C amino acid and the nascent polypeptide chains were labelled. Following this, the cells were broken open and the cytoplasmic extract was sedimented on a sucrose gradient. The nascent polypeptide chains are found in the polyribosomal fraction but not attached to the single ribosomes[16]. Various fractions along the gradient were examined in the electron microscope to measure the size of the polysomal units. The numbers shown on the bottom of Fig. 4 refer to the number of ribosomes. Although there is a peak in the polysomal distribution appearing at pentamers or hexamers, it extends out to very large polysomes which contain more than fifty ribosomes near the bottom of the gradient. This is in sharp contrast to the very circumscribed distribution contained in the reticulocyte and it is interpreted as a demonstration of the fact that there are large varieties of messenger RNA lengths found in a cell making a variety of proteins.

5. The attachment of labelled ribosomes to unlabelled polysomes during *in vitro* incubation has been demonstrated in lysates from HeLa[13] as well as in the reticulocyte system[17]. The attachment of ribosomes is stimulated by conditions which favour protein synthesis. In a system such

as the HeLa extract in which there are a variety of polysome sizes, the number of ribosomes attaching to the polysomal units is proportional to the number of messenger RNA ends which are found in the solution. Thus, for example, after a brief period of incubation, twice as many ribosomes are attached to a polysome containing five ribosomes as are found attached to an equal number of ribosomes in a fraction containing

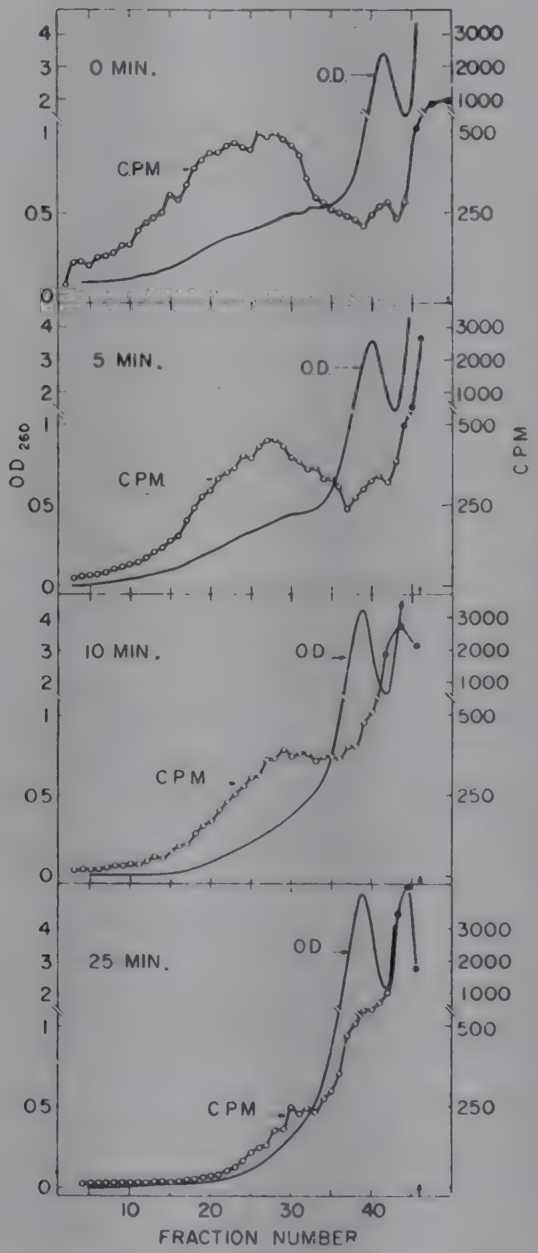


Fig. 5—Sucrose density gradient analysis of HeLa cell polysome incubation[13]: Polysomes of HeLa cells were labelled with ^{14}C amino acids, the cells were broken open and the polyribosomes were pelleted gently. The radioactive supernatant was decanted and a non-radioactive supernatant was added to the pellet. Then the labelled polysomes were resuspended and incubated with an energy generating system and with amino acids. At 0 min. it can be seen that most of the radioactivity is in the polysomal peak. As time proceeds, the radioactivity in the polyribosomal peak decreases and 70% of it is transferred to soluble protein at the top of the gradient. Associated with this release of polypeptide chains there is also a decrease in the optical density of ribosomes in the polysome peak. These ribosomes accumulate in the single ribosome peak

polysomal clusters with ten ribosomes. Although this proportionality of attachment is valid for polysomes containing up to ten or twenty ribosomes, it has not yet been demonstrated unambiguously for much larger polyribosomal structures. Some of the latter are undoubtedly polycistronic in that the messenger RNA codes for more than one protein molecule[18,19].

6. The detachment of ribosomes from polyribosomes under conditions of *in vitro* incubation has been demonstrated in liver cell lysates[20] and in HeLa cell lysates as shown in Fig. 5. HeLa cell polysomes were labelled *in vivo* with ^{14}C amino acids and the polysome fraction was isolated by centrifugation. The labelled polysome was then resuspended in an unlabelled supernatant and at zero time the sucrose gradient shows that most of the labelled nascent protein is found in the polysomal fraction which has been degraded slightly by this preparative procedure. However, as incubation proceeds (Fig. 5), the optical density of the polyribosomal peak decreases while the optical density of the single monomeric ribosome peak increases. This occurs during *in vitro* incubation because the rate of ribosomal attachment to polysomal units is less than the rate of detachment. Consequentially, the ribosomes run off the structure as the incubation proceeds.

7. Associated with the detachment of ribosomes during *in vitro* incubation, there is also a detachment of polypeptide chains[13]. This is seen in Fig. 5 where 70% of the radioactivity originally in the polysomal fraction has left the polysomes after 25 minutes of incubation and is then found as soluble protein at the top of the gradient. If this transfer were the result of the action of ribonuclease in the system, the radioactivity would be transferred to the single ribosome region but not liberated as soluble protein. These experiments show that the release of the polypeptide chain is dependent upon an ATP generating system and other conditions which favour protein synthesis[13].

The experiments which are outlined above all serve to reinforce the general picture of polyribosomal function which is described in Fig. 3. However, this description leaves a great many unanswered questions. For example, what is the mechanism of ribosomal movement along the messenger, what is the mechanism of ribosomal attachment and detachment to the messenger RNA, or what is the mechanism of polypeptide chain initiation or completion ? There are very few experimental answers at the present time to most of these questions. However, some insight into the mechanism has been provided by a study of the sRNA molecules which are associated with the polyribosomes.

Soluble RNA of Polysomes

Soluble RNA molecules have been shown to participate in two distinctive functions during protein synthesis: they serve as the vehicle for transporting amino acids to the ribosomes and they are attached to the growing polypeptide chains[12, 21]. Several investigators have shown that sRNA molecules[22–24] will bind to ribosomes *in vitro* but only recently in a study of the sRNA in intact reticulocytes has it been possible to differentiate ribosomal from polysomal sRNA[25]. The reticulocyte has no nucleus and does not manufacture any RNA; however, it is possible to label selectively the sRNA by exposing intact cells to radioactive adenine. The sRNA molecules slowly incorporate the adenine into the adenosine end of the molecule. After a period of incubation, the cells are broken open and analysed on a sucrose density gradient. Labelled sRNA is found associated with both the single ribosomes and with the polysomes. The results can then be plotted in terms of radioactivity per optical density unit (OD_{260}) in the single ribosomal peak and the polysomes. Knowing the specific activity of the soluble RNA the radioactivity can be converted to the number of sRNA molecules while the optical density can be converted

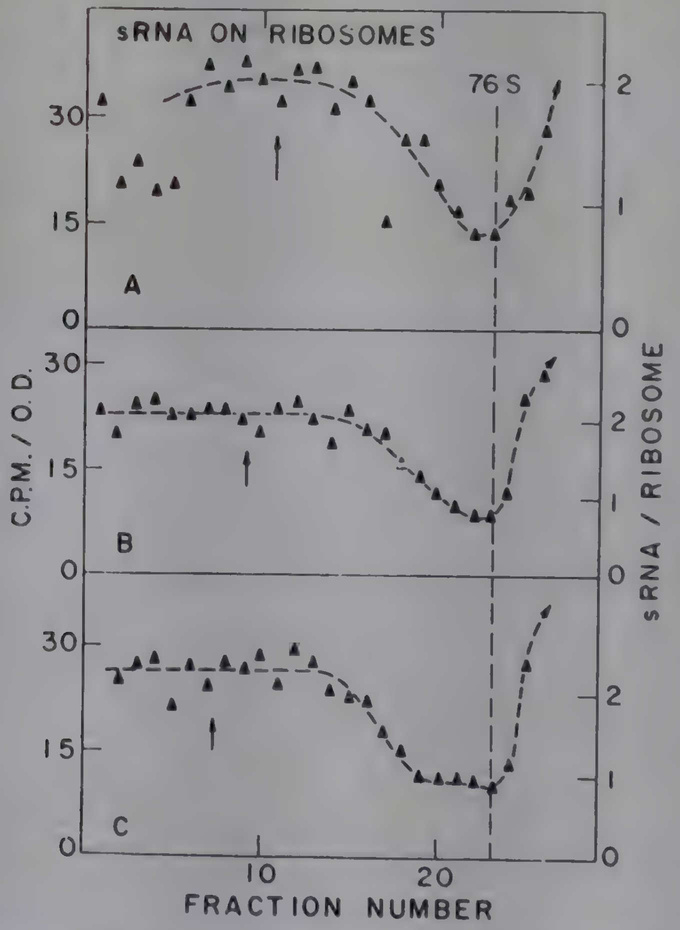


FIG. 6—SUCROSE DENSITY GRADIENT ANALYSIS OF RETICULOCYTE POLYSOMES ON WHICH THE sRNA HAS BEEN LABELLED BY RADIOACTIVE ADENINE[25]: Radioactivity and polysomal optical density were measured on a sucrose gradient and the ratio of radioactivity over optical density is plotted here. The vertical dashed line labelled 76s represents the position of single ribosomes while the arrow represents the position of the polysomal optical density maximum in three different experiments, A through C. It can be seen that the ratio of radioactivity over optical density drops to a low value in the region of single ribosomes and then rises to a plateau in the polysome region. The ratio is also plotted as sRNA molecules per ribosome

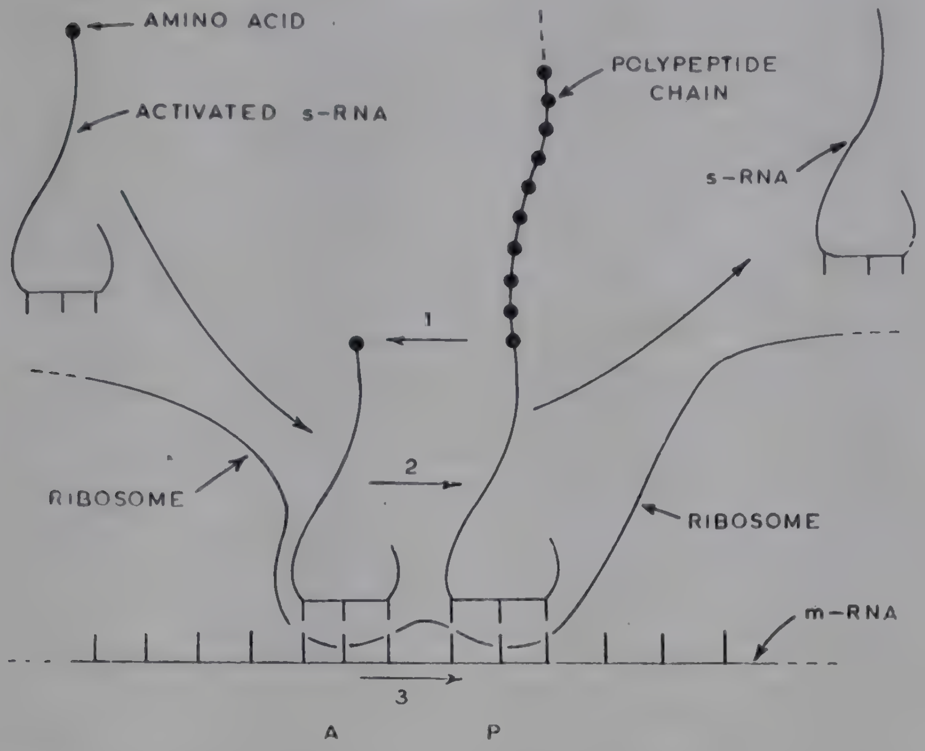


FIG. 7—A SCHEMATIC DIAGRAM SHOWING THE ACTION OF THE TWO sRNA SITES ON THE RIBOSOME DURING PROTEIN SYNTHESIS: Site A holds the amino acid sRNA which is about to be added to the polypeptide chain. Site P holds the sRNA with the growing polypeptide chain. The growing polypeptide chain is transferred to the sRNA on site A, which allows the sRNA on Site P to be released. After that, the sRNA and the messenger RNA are both transferred together from site A to site P. The latter is a schematic representation of the ribosomal movement along the messenger strand

to the number of ribosomes. The results of three experiments are shown in Fig. 6, where the vertical dashed line represents the sedimenting position of the single ribosomes and the arrows represent the optical density peak of the polysomes which are sedimenting to the left. It can be seen that the single ribosomes have one or slightly less than one sRNA molecule attached to each ribosome, while the polysomal peak has two sRNA molecules per ribosome. Experiments in which unlabelled sRNA molecules are added or in which the magnesium ion concentration is lowered have demonstrated that the sRNA molecule attached to the single ribosomes is readily exchangeable and less tightly bound than are the two sRNA molecules attached to the polysomes[25]. These experiments have been interpreted as suggesting that there are two sRNA sites on the ribosome, one of which holds the growing polypeptide chain (site P), whereas the other contains the sRNA-amino acid which is to be added next to the growing polypeptide chain (site A). This is illustrated schematically in Fig. 7, where the solid circle represents an amino acid and the curved structure represents the sRNA molecule with its three coding nucleotides. The mechanism of protein synthesis in this scheme consists of a coupling of action between the two sites, A and P. Three steps have to occur in a cyclic fashion. First of all, the growing polypeptide chain must be transferred from the sRNA on site P to the sRNA on site A, followed by the release of the sRNA on site P. Secondly, the sRNA on site A must be transferred to site P coupled with. Thirdly, a corresponding transfer of the triplet of coding messenger nucleotides from site A to site P. The joint movement of both the sRNA and the messenger from site A to site P may then be looked upon as the basic operation whereby the ribosome moves relative to the messenger strand. Following this, site A is open for the next amino acid sRNA and the operation repeats itself. Fig. 7 describes these operations in a schematic fashion. However, further experimentation will be necessary to supply the physical basis for this diagrammatic description.

DISTRIBUTION OF POLYRIBOSOMES

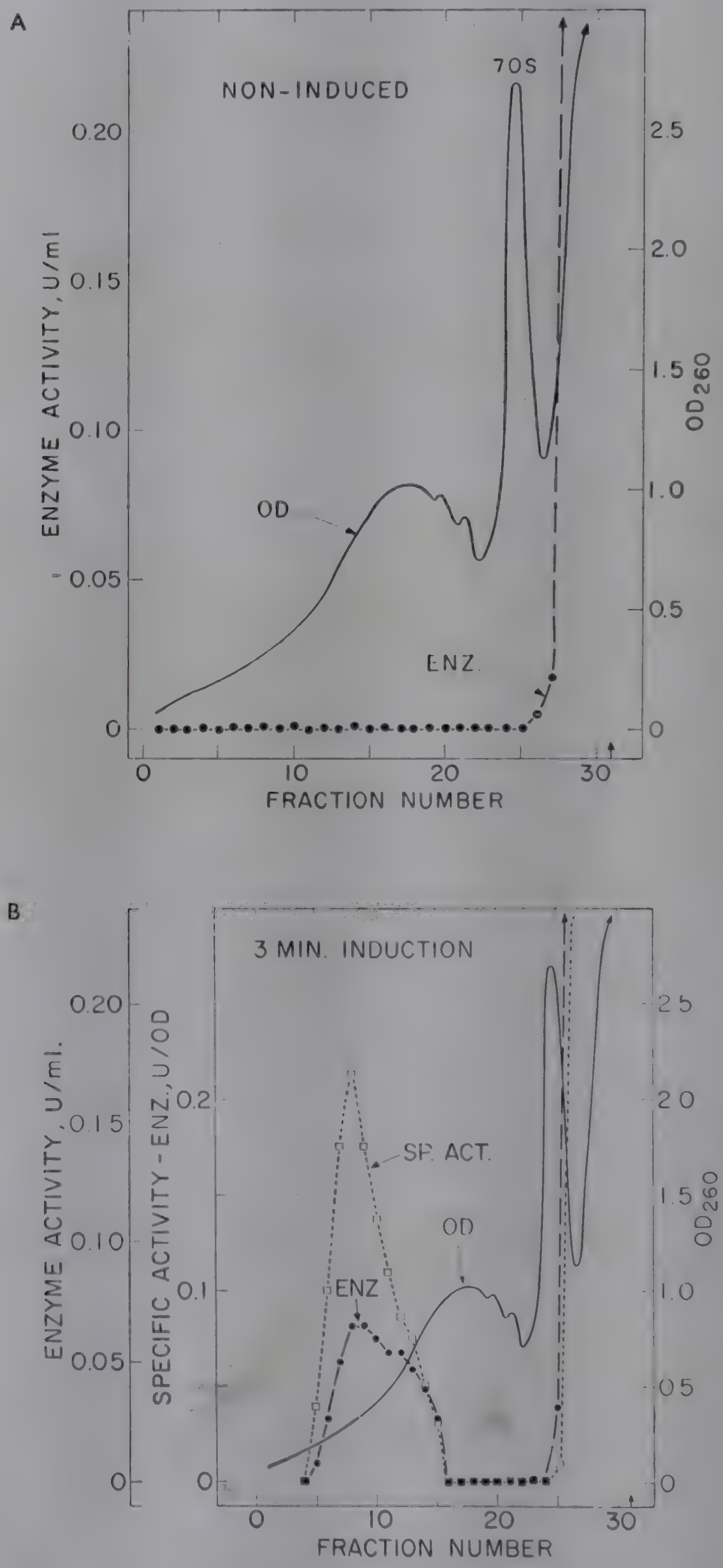
It is likely that most protein synthesis takes place on polyribosomes rather than on single ribosomes. Most of the polysomal structures which have been looked at in the electron microscope seem to show a spacing between adjacent ribosomes similar to that which is seen in the reticulocyte polysomes where five ribosomes are found on a messenger which is coding for about 150 amino acids. Thus, the messenger RNA linking a ribosome to its nearest neighbour codes for approximately 30 amino acids. Most proteins have polypeptide chains with more than 30 amino acids, hence, it is likely that the synthesis of most proteins will take place on polyribosomal structures. It may turn out that physiological variations especially in microorganisms will show that inter-ribosomal spacings are variable, but there is no indication of this at the present time.

Polyribosomal structures are somewhat fragile; accordingly, great care must be exercised in opening cells in order to retain them in an intact form. When cells are broken open under proper conditions, most of the protein synthesis has been demonstrated on polysomal structures. In mammalian systems, polysomes have been reported in liver[26] as well as in HeLa cells and the specialized reticulocyte cells. In the homogenized chick embryo, all of the proteins are synthesized on polyribosomal structures[27]. They can also be demonstrated in individual tissues, such as brain, liver or connective tissue. Polysomes have also been reported in plant tissues[28], in cellular slime molds[29] as well as in a variety of bacteria[30,31]. It seems quite likely that the polyribosomal mechanism of protein synthesis is ubiquitous.

In thin slices of cells and tissues, ribosomes are often seen in association with the sheet-like endoplasmic reticulum. These membraneous structures are not found in all cells. For example, bacteria, reticulocytes or rapidly growing mammalian cells such as HeLa or young embryonic cells do not contain an endoplasmic reticulum. However, in many highly differentiated cells, a large number of ribosomes are found attached to the endoplasmic reticulum where they are often seen occurring in linear arrays, double rows, whorls or rosettes[32]. When these cells are gently broken open, the endoplasmic reticulum can be dissolved through the use of the steroid deoxycholate which disperses the lipid containing material of the membrane. Following this treatment, polysomal material can be demonstrated on a sucrose gradient. Thus, it is quite likely that the ribosomes are connected to long messenger RNA strands at the same time that they are organized in various linear arrays attached to the membrane. We may speculate on the mechanism of polyribosomal protein synthesis in a cell in which the ribosomes are presumably attached to the endoplasmic reticulum. At the present time we have no evidence concerning the mobility of the ribosomes on this membrane. It is possible that the ribosomes do not move about rapidly on the membrane but remain attached for some time. However, since the ribosomes are usually found not in a random configuration but in linear arrays, it is reasonable to suppose that the long messenger RNA molecule is passing along these chains. In this interpretation, the messenger molecules are released from the nuclear material and approach one side of the endoplasmic reticulum. The attachment end of the messenger strand engages a ribosome, and then passes through as protein synthesis is initiated. When the attachment end is liberated, it engages on to an adjoining ribosome and is then pulled through that structure as well. A continuation of this process would thus involve a threading of a messenger strand along the ribosomal arrays attached to the membrane of the endoplasmic reticulum. Presumably, when the messenger passes through the ribosome, there is a release of completed protein. Endoplasmic reticulum is often associated with excretory activities of the cell, and the products of the protein synthetic reaction are seen to accumulate in the lumen of the endoplasmic reticulum. This implies that although activated soluble RNA molecules and messenger RNA approach the reticulum from one side, the protein product of this interaction is released on the opposite side of the membrane. The mechanism for this process is completely unknown at the present time. Nonetheless, it may be reasonable to believe that the linear arrays of ribosomes seen on the endoplasmic reticulum actually reflect the functional organization of the ribosomes when they are associated with a linear messenger strand.

Synthesis of Specific Proteins on Polyribosomes

The polyribosomal distribution in the reticulocyte is quite narrow because it is manufacturing a single protein hemoglobin. As one looks at a broader distribution of polyribosomes such as that seen in a bacterial cell, for example, one would anticipate that different proteins would be made on different sizes of polyribosomes if a particular size of messenger RNA was associated with a particular protein. It has been possible to demonstrate this in special cases in which there are distinguishing features which make it possible to identify particular proteins. A study of this type has been carried out with the induced enzyme β-galactosidase. The pro-


A
NON-INDUCED
70S
OD
ENZ.
ENZYME ACTIVITY, U/ml
0
0.05
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0
0.5
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3 MIN. INDUCTION
SP. ACT.
OD
ENZ
ENZYME ACTIVITY, U/ml.
0
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SPECIFIC ACTIVITY - ENZ., U/OD
0
0.1
0.2
OD260
0
0.5
1.0
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2.0
2.5
0
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30
FRACTION NUMBER

duction of this enzyme is stimulated by the addition of inducer molecules and according to the analysis by Jacob and Monod[1], this is associated with the production of a new messenger RNA molecule. β-Galactosidase activity has been found associated with ribosomes[33] and a kinetic analysis of this material suggested that it is a precursor of the soluble protein[34]. It is possible to assay for the induced enzyme directly on polyribosomes[35]. An example of this type of analysis is shown in Fig. 8. In Fig. 8A, the ribosome and polysome distribution of non-induced *Escherichia coli* is shown. The sharp 70 s peak represents the single ribosomes and the polyribosomes are further to the left. Individual fractions have been assayed for the presence of β-galactosidase and no activity is present except for a small amount at the top of the gradient which represents the basal level of the enzyme in the absence of an inducer. When inducer is added, however, the production of β-galactosidase is initiated and, associated with this, a rise in enzymatic activity can be detected in a large polyribosomal fraction (Fig. 8B). The enzymatic activity per optical density unit rises to a sharp peak which is associated with polyribosomes containing 40 or 50 ribosomes as determined by electron microscope studies. A kinetic analysis of the polyribosomally bound enzyme shows that this activity rises very rapidly during the onset of enzyme induction and reaches a steady state when the production of new enzyme has reached a maximum steady state value. This confirms the results of the earlier analysis[34] which concluded that the ribosomally bound activity is a kinetic precursor of free β-galactosidase activity in the cell.

The enzymatic activity which is attached to polyribosomes has all of the characteristics of nascent protein. Thus, if a peak polysomal fraction such as shown in Fig. 8B is rerun on the sucrose gradient, it sediments with the same speed. However, treatment of an aliquot with ribonuclease converts the polysomes into single 70 s ribosomes which retain all of the enzymatic activity. Thus, destruction of the messenger RNA strand converts the polyribosomal material into single ribosomes which retain their attached enzymatic activity in the same way that they retain the radioactivity of the nascent protein in experiments carried out with ^{14}C-labelled amino acids.

The fact that enzymatic activity is associated with polyribosomes and that it behaves like incomplete protein molecules suggest that the growing polypeptide chains are folded into native configurations involving both secondary and tertiary structure. Thus, the schematic diagram in Fig. 3 showing extended polypeptide chains must be modified.

←FIG. 8—SUCROSE DENSITY GRADIENT ANALYSIS OF *Esch. coli* POLYSOMES OBTAINED FROM (A) NON-INDUCED AND (B) INDUCED CELLS[35]: The solid line represents the optical density with the single ribosomal peak labelled 70 s. The line with solid circles represents the β-galactosidase enzymatic activity assayed on various fractions. The dashed line represents the specific activity or enzymatic activity per ribosomal optical density unit. Although there is no enzymatic activity assayed on polysomes in non-induced cells, three minutes after induction the cells show enzymatic activity associated with a fraction of longer polysomes

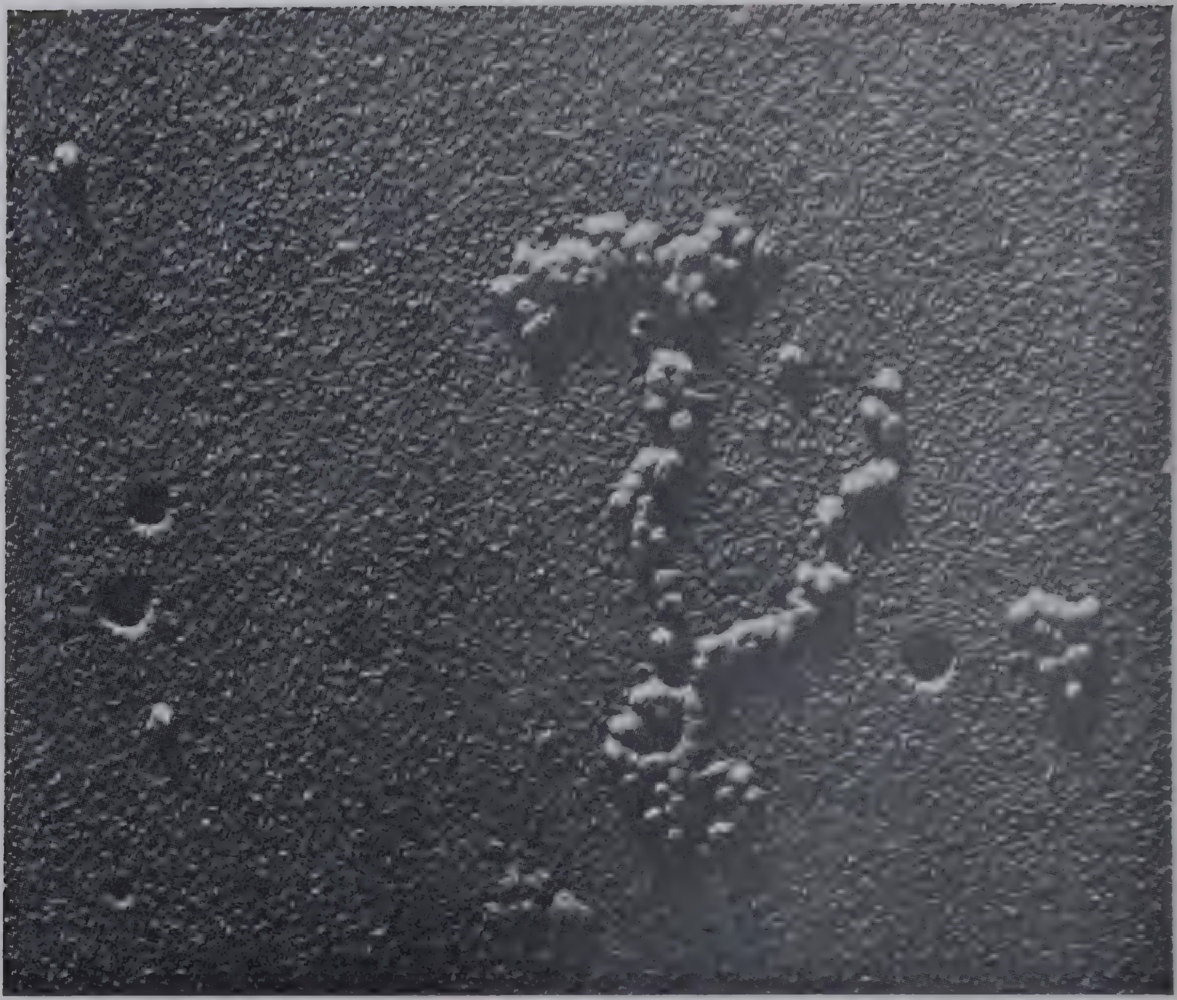

FIG. 9—AN ELECTRON MICROGRAPH OF POLIO INDUCED POLYRIBOSOMES[16]: The large irregularly shaped polyribosomal strand is an example of that which is seen about 3 hr after polio virus infection in actinomycin D treated HeLa cells. There are between 50 and 70 ribosomes attached to the single polyribosomal strand. The overall shape of the polyribosomal cluster is not significant, since it is strongly affected by drying forces on the electron microscope grid. The material here has been shadowed with platinum at a 5:1 angle

We can estimate the size of the messenger RNA associated with β-galactosidase induction from the size of the polysome containing 40 to 50 ribosomes. The pentaribosomal unit in hemoglobin is believed to contain a messenger with 450 nucleotides; hence, a 50 ribosomal unit should contain 4500 nucleotides with a total molecular weight slightly over 1.7 million. The β-galactosidase molecule is made of four subunits each with a molecular weight near 125,000 (Ref. 36). The information required to produce this subunit would be found in a messenger RNA of approximately 1·2 million. Thus, the β-galactosidase polyribosome is larger than it should be if it were only coding for the β-galactosidase protein.

Associated with the induction of β-galactosidase is the production of other enzymes, permease-acetylase[1]. It has been possible to demonstrate that the messenger strand which holds the β-galactosidase polysome together codes for both the galactosidase and the permease activities[19]. This was shown directly by measuring the size of the polyribosome in the normal wild type organism which produces both β-galactosidase and permease proteins. However, in mutant organisms which have a large deletion mutation in the permease region, smaller galactosidase polysomes are produced than that seen in the normal wild type. This can be explained by recognizing that the messenger strand contains information for making both of these molecules. This is an example of a polycistronic messenger which codes for more than one type of protein.

Another type of polycistronic messenger strand is seen during viral infection. HeLa cells can be treated with actinomycin D which inhibits the production of endogenous messenger in the cell[37]. However, polio virus production is not inhibited because polio is an RNA virus and these are not inhibited by actinomycin D. After infection of actinomycin D treated HeLa cells with polio virus, polio polysomes are seen which are producing protein molecules associated with polio infection. When these polysomes are examined in the electron microscope, they are very large and contain from 60 to 80 ribosomes in each cluster[16]. An example is shown in Fig. 9. The polio virus contains RNA with a molecular weight of 2 million and the size of the largest polyribosome seen in polio infection is consistent with the interpretation that the entire polio RNA is acting as a messenger. Several new proteins are found subsequent to polio virus infection, including a new RNA polymerase as well as various structural proteins associated with the viral coat. Thus, the polio RNA strand must contain information for making more than one protein and it is accordingly polycistronic. An electron microscopic analysis of the early stages of polio infection reveals ribosomes which are widely spaced on the polio virus RNA, thereby suggesting that the various cistrons are manufacturing proteins independent of each other rather than in a synchronous fashion[16].

The formation of the fibrous protein collagen has been studied on the polyribosomes of the embryonic chick[37]. Collagen is a fibrous protein which consists of three polypeptide chains coiled about each other, each of which has a molecular weight of approximately 100,000. Collagen has the unique property of containing the amino acid hydroxyproline which is not present in any other protein. This makes it possible to study the formation of collagen on polyribosomes by measuring the distribution of hydroxyproline on nascent protein attached to polyribosomal material. The hydroxyproline of collagen is formed from the amino acid proline. Accordingly, these experiments are carried out by injecting a chick embryo with labelled proline and then assaying the resultant polyribosomes for hydroxyproline. The results indicate that collagen formation occurs on extremely large polyribosomes which sediment very rapidly. They are somewhat polydisperse but it has been estimated that the smallest size contains approximately 100 ribosomes. Based on the calibration used with the study of hemoglobin formation in reticulocytes, we can estimate that the 100 ribosomal unit has enough messenger RNA information to code for all three of the collagen polypeptide chains. These polysomes are partially resistant to ribonuclease, in that they do not all break down to yield single ribosomes. This suggests that the triple-chained helical collagen molecule is folded together on the polyribosomes as the chains are formed[27]. However, further work must be done in this system before the mechanism of collagen formation can be fully elucidated.

Formation and Destruction of Messenger RNA

Very little is known in general about the control of messenger RNA formation even though for certain inducible enzymes, the inducer-repressor system has been described in some detail[1]. However, the generality of the mechanism is completely unknown at the present time. Equally little is known concerning the detailed mechanism of messenger RNA destruction. Experiments in bacterial cells treated with actinomycin D have shown that there is a very rapid turnover of messenger with an average half life of a few minutes at 37°C. (Ref. 38). The half life has not been measured in detail for very many individual messenger RNA strands.

Even though there is a lack of experimental information, some reasonable inferences can be drawn regarding the mode of messenger RNA breakdown. We can postulate some properties which should be found in the breakdown mechanism. The destruction should involve an exonuclease digestion which attacks from one end of the molecule. An endonuclease attack would lead to the fragmentation of the polyribosomal structure which would then give rise to non-functional fragments of protein molecules. The most efficient system would be destruction from one end. Furthermore, we infer that the direction of exonuclease digestion must be parallel to the direction of ribosomal movement along the messenger RNA strand. If the exonuclease were to initiate the digestion of the messenger RNA from the detachment end of the messenger strand, then the continued flow of ribosomes over the truncated messenger strand would result in a continued production of abnormal proteins which are increasingly deficient at the carboxyl end of the polypeptide chain. It seems somewhat implausible that nature would utilize a mechanism which is so inefficient. Thus, in a mechanism of the type postulated, the attachment of ribosomes to the messenger is stopped but those ribosomes which are already attached will simply continue to migrate along the messenger normally and complete the synthesis of each individual protein. This implies that there may be a definite attachment site on the messenger, and that its destruction prevents the further attachment of new ribosomes.

If a mechanism of this type is involved in the destruction of messenger RNA, then the hydrolytic exonuclease enzyme may be regarded as competing with a ribosome for the attachment at the end of the messenger strand.

If the ribosome attaches, protein synthesis is initiated and the messenger strand is not destroyed. Alternatively, when the ribosome moves down the strand, the end is then uncovered and again the exonuclease enzyme has an opportunity to initiate the destruction of the ribosomal attachment site. In a system of this type, it is quite easy to imagine an additional facet for the control of messenger half life. There may be a sequence of nucleotides at the end of the messenger strand which, by the composition of its nucleotides, determines the binding affinity for the hydrolytic exonuclease. If this is so, the messenger RNA is formed not only with sequence information for manufacturing the molecule but also with information which regulates the rate at which the messenger is destroyed.

It must be emphasized that at present we have very little information concerning the manner in which directions for assembly are encoded into the sequence of nucleotides of the messenger RNA strand. We do not know if specific nucleotides are needed for the attachment of ribosomes, for the initiation of chain synthesis or the completion and detachment of the polypeptide chains. However, the existence of polycistronic messenger molecules clearly points out the fact that some type of regulation can occur on the polysomal level. Further experiments will undoubtedly reveal the nature of this regulatory system.

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Studies on the Amino Acid Code: The Ambiguity in Translation*

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Several lines of evidence support the hypothesis that the amino acid sequence of a protein is determined by the nucleotide sequence of the DNA segment, i.e. the cistron specifying that protein[1].

The correspondence of the genetic map with the sequence of blocks of nucleotides in DNA was demonstrated earlier[2]. The colinear relationship between the genetic map and protein was proved recently[3,4]. It was shown that the positions of amino acid replacements in a given protein were in the same relative order as the order of the mutationally altered sites of the corresponding mutants in the cistron specifying the protein in question[3]. It was also demonstrated that the genetic recombination values are representative of the distances between amino acid residues[3].

In the first step of protein synthesis—transcription—DNA serves as the template for the RNA nucleotidyl transferase catalysed synthesis of m-RNA[5]. Each m-RNA is, as far as its base sequence is concerned, a complementary copy of one strand of one or more cistrons[6–8].

The amino acid sequence is assumed to be encoded as a sequence of nucleotide triplets—codons—in m-RNA, each triplet representing an amino acid[9].

Since no single case has been reported so far in which two adjacent amino acids were replaced in a single step mutation (see the studies on the amino

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acid replacements in the coat protein of nitrous acid induced tobacco mosaic virus mutants by H. G. Wittmann in this volume), it is assumed that no nucleotide participates in the determination of more than one amino acid, i.e. that the triplets do not overlap[10]. The evidence for the triplet code is of genetic nature[9]. In the second complex step of protein synthesis—translation—one or more ribosomes attach to m-RNA (see the contribution of A. Rich in this volume). The linking of amino acids occurs on the surface of these m-RNA ribosome complexes. For this to take place, amino acids must first be activated and linked by a specific amino acyl-t-RNA synthetase to the terminal adenosine moiety of an amino acid specific t-RNA[11–13].

Supposedly, somewhere along the nucleotide sequence of a particular t-RNA there is a segment called adaptor complementary to a triplet specifying the same amino acid for which the t-RNA in question is specific[1]. The ribosome bound m-RNA is translated by the t-RNA triplet by triplet. The assembly of amino acids starts at the amino terminal end of the polypeptide chain and proceeds amino acid by amino acid toward the carboxy terminal end[14,15]. The addition of an amino acid to the peptide chain is believed to occur in the following way: the terminal carboxyl group of the growing polypeptide chain is always attached to t-RNA[16,17]. This t-RNA binds it to the ribosome bound m-RNA presumably through hydrogen bonds between the adaptor and the triplet which is complementary to it. Since protein synthesis occurs under conditions in which the strength of binding between two trinucleotides is not sufficient to hold them together, it is assumed that some other component must reinforce this interaction. The next amino acyl-t-RNA is attached to the adjacent triplet. The alpha amino group of this amino acid displaces the first t-RNA from ester linkage with the polypeptide by forming a new peptide bond with the polypeptide. The completed peptide chain is released from the ribosome after splitting its linkage to t-RNA. The transfer of the amino acid residue from t-RNA to protein requires GTP, and at least two enzymes are involved in these steps[18–21].

The list of amino acids with the triplets specifying each in m-RNA is the amino acid code. It may be assumed that m-RNA contains in addition, sequences not specifying amino acids but determining for example the start of a new peptide.

The possibility for the experimental deciphering of the code was opened by the experimental verification of the m-RNA concept of Jacob and Monod[22–24]. It was demonstrated, namely, that old ribosomes can attach to new m-RNA and participate in the synthesis of new protein, information for which is encoded in the new messenger[23]. This proved that ribosomes were non-specific structures that synthesize at a given time the protein specified by the messenger to which they happen to be attached. It was a logical extension of this finding to provide ribosomes in cell-free amino acid incorporating systems with artificial messengers (synthetic polynucleotides) with the aim of using this system as a tool for code deciphering. Cell-free amino acid incorporating systems were available, and synthetic polynucleotides could be prepared with polynucleotide phosphorylase[25]. The simplest messenger is a homopolynucleotide which, if active at all, should direct the formation of a homopolypeptide. Nirenberg and Matthaei studying the effect of RNA from various sources on amino acid incorporation in a cell-free *Esch. coli* system were the first to find that poly U promoted the formation of polyphenylalanine[26]. A random copolynucleotide containing several kinds of nucleotides, if active, should direct the formation of a polypeptide containing several kinds of amino acids. Indeed, by introducing the use of copolynucleotides of random sequence (as well as of additional homopolynucleotides), the deciphering of the code was extended in our laboratory to the 19 other amino acids[27]. It was shown that the base composition of the random copolynucleotides used as messengers determines the amino acid composition of the peptide product formed in the *in vitro* system[28]. Evidence was provided that the product, formation of which was promoted by the synthetic messenger, is indeed a polypeptide[29]. In the first part of this communication we will attempt to summarize the results in code deciphering obtained so far.

At the beginning of this study, a disturbing result was found: poly U, a homopolynucleotide consisting of only one kind of triplet, in addition to promoting incorporation of large amounts of phenylalanine, promoted the incorporation of a small, but significant amount of leucine too[27,30,31]. Thus, the UUU triplet acted in an ambiguous way. Recent experiments on the nature of this ambiguity and its possible significance form the second part of this communication.

AMINO ACID INCORPORATING SYSTEM

The system consisted of *Esch. coli W* ribosomes and high speed supernatant which contained t-RNA, the amino acyl-t-RNA synthetases, and the enzymes involved in the final steps of the synthesis of the polypeptide chain[27]. It was supplemented with amino acids, ATP, GTP, salts, and t-RNA[27,32,33]. Polynucleotide phosphorylase, specific activity 30-60, was used to prepare polyribonucleotides[34]. The base ratio of the copolymers synthesized closely reflected the nucleoside diphosphate composition of the incubation mixture in which they were prepared[35].

To observe the effect of polynucleotides on the incorporation of a given amino acid into protein, this amino acid labelled with ^{14}C was included in the incubation mixture along with the remaining 19 non-labelled amino acids in experiments on code triplet assignment. In experiments on ambiguity no unlabelled amino acids were added. Incorporation of the labelled amino acid into protein was measured in the usual way by acid precipitation,

TABLE 1—Amino Acid Incorporation in *Esch. coli* System with Various Polynucleotides*

	Polynucleotide†									
	AC (5 : 1)		AC (1 : 5)		UC (5 : 1)		UC (4·3 : 5·7)		UC (1 : 5)	
Amino Acid	none	80 µg./ml.	none	320 µg./ml.	none	160 µg./ml.	none	320 µg./ml.	none	320 µg./ml.
Asparagine	47	1146	27	98	..	..	..	..	..	..
Glutamine	39	1117	31	101	..	..	..	..	..	..
Histidine	282	576	28	343	..	..	..	..	..	..
Leucine	..	..	..	..	74	3368	49	4100	69	659
Lysine	60	4615	12	26	..	..	..	..	..	..
Phenylalanine	..	..	..	..	49	14860	96	4516	79	159
Proline	14	342	8	1350	21	784	47	7785	51	2880
Serine	..	..	..	..	46	3540	41	4035	44	586
Threonine	44	1250	22	301	..	..	..	..	..	..

*$\mu\mu$moles/mg. ribosomal protein. The following precipitating reagents were used: 5% trichloroacetic acid in the experiments with poly UC (5 : 1); 20% trichloroacetic acid in those with poly UC (4·3 : 5·7), UC (1 : 5), AC (1 : 5); and 0·25% sodium tungstate in 5 per cent trichloroacetic acid in those with poly AC (5 : 1). The results presented in this table are average values of an experiment done with duplicates. Similar results were obtained in at least one other experiment.

†The base ratios given in parentheses are the ratios of nucleoside diphosphates used in the preparation of the polymers except for poly UC (4·3 : 5·7). In this case the actually determined base ratio is given (the polymer was prepared with UDP and CDP in 1 : 1 ratio). The base ratios of poly UC (5 : 1) and AC (5 : 1), as actually determined, were 4·7 : 1 and 4·9 : 1, respectively.

washing, and counting of the radioactivity of the precipitate. The precipitating agents used varied, depending upon the polynucleotides employed (and hence on polypeptide product expected), and are listed in the legend to Table 1[27,35,36]. The small incorporation observed in the absence of added messenger was reduced by preincubation of the ribosomes and superratant.

Effect of Homopolymers

Poly U directs the incorporation of phenylalanine[26], and to a lesser extent, leucine; poly A the incorporation of lysine[35]; and poly C the incorporation of proline into acid insoluble products[36]. Poly G was not tested because it is difficult to obtain and has a multi-stranded structure[37]. Inosinic acid residues in copolymers behave as guanylic acid residues with regard to coding properties[38]. Therefore, poly I was tested for promoting amino acid incorporation; it was inactive. It remains to be seen whether the lack of activity of poly I is solely due to the triple-stranded structure of this polymer[39].

EFFECT OF RANDOM COPOLYMERS

To illustrate the method of assigning code triplets to amino acids by using random copolymers[27] we will discuss results with two kinds of copolymers. Table 1 shows data on the promotion of amino acid incorporation by copolymers of A and C, and of U and C[28]. The specificity of action of these copolymers is obvious. They were tested with all 20 amino acids and had no effect on the incorporation of the amino acids not listed in Table 1. Poly AC promotes the incorporation of 6 amino acids, poly UC that of 4 amino acids. There is only one amino acid, incorporation of which is promoted by both polymers. It follows that all amino acids, incorporation of which is promoted by poly AC, must have at least one triplet consisting of A and/or C only, and all amino acids, incorporation of which is promoted by poly UC, must have at least one triplet consisting of U and/or C only. The results were interpreted quantitatively, with the basic assumption that polymers made by polynucleotide phosphorylase are random in sequence. Actually, the nearest neighbour distribution of such polymers, i.e. poly AU (1 : 1) and poly ACGU (1 : 1 : 1 : 1) has been shown to be random[40,41].

With this assumption the frequency of particular triplets (sequences of three nucleotides) in such a copolymer, i.e. the triplet composition, can be calculated and matched with the amino acid composition of the product (polypeptides) the formation of which is promoted by the copolymer[27]. This amino acid composition is calculated from the extent of incorporation of the various amino acids.

Actually, if data are available on the promotion of the incorporation of only two amino acids by a copolymer of proper base ratio, and the composition of the triplet of one is known, the composition of the triplet of the other can be calculated.

Three points may be made:

1. This type of analysis is not possible with a natural m-RNA such as viral RNA, because at present the sequence of such RNA is unknown and it is surely not random.

2. The amino acid composition of the product is independent of both the rate and the yield of the reaction and is not affected by the action of exonucleases on the copolymer which promotes the incorporation. These exonucleases may affect experiments with non-random copolymers.

3. With the random copolymers one can determine only the nucleotide composition of a triplet but not the sequence within that triplet.

The triplet assignments are in agreement with a triplet code, but in themselves do not prove the triplet nature of the code.

EXAMPLE OF CALCULATION OF THE TRIPLET COMPOSITION OF A RANDOM COPOLYMER AND OF TRIPLET ASSIGNMENTS

Poly AC (5:1) contains 8 kinds of triplets but promotes the incorporation of only 6 amino acids. What is the triplet composition of this copolymer ? On the average 5 out of every 6 nucleotides in it are A, one out of every 6 nucleotides is C.

The frequency of the 3A triplet is

$$\frac{5\times5\times5}{6\times6\times6}=\frac{125}{216}$$

i.e. on the average out of every 216 triplets there are 125 3A triplets. We call the frequency of the 3A triplet 100 and express the frequencies of all other triplets as percentages of the frequency of the 3A triplet.

The frequency of each of the three 2A1C triplets, AAC, ACA, CAA, is

$$\frac{5\times5\times1}{6\times6\times6}=\frac{25}{216}$$

i.e. 20%.

The frequency of each of the three 1A2C triplets, ACC, CAC, CCA, is

$$\frac{5\times1\times1}{6\times6\times6}=\frac{5}{216}$$

i.e. 4%.

The frequency of the 3C triplet is

$$\frac{1\times1\times1}{6\times6\times6}=\frac{1}{216}$$

i.e. 0·8%.

The triplet composition is expected to be reflected by the amino acid composition of the polypeptide, the formation of which is promoted by this polymer. Table 1 shows data on the actual amino acid incorporations by the *Esch. coli* system as promoted by various polynucleotides. From the homopolymer experiments mentioned previously, the 3A triplet is known to code for lysine and the 3C triplet to code for proline. Poly AC (5 : 1) (Table 2) promotes the incorporation of asparagine, glutamine, and

TABLE 2—Data for Code Triplet Assignments (Copolymers of A and C)

Poly AC (5 : 1)

Amino acid	Calculated triplet frequency				Sum of calculated triplet frequencies	Amino acid incorporation*
	3A	2A1C	1A2C	3C		
Asparagine	..	20	..	..	20	24·2
Glutamine	..	20	..	..	20	23·7
Histidine	..	..	4·0	..	4	6·5
Lysine	100	..	..	..	100	100
Proline	..	..	4·0	0·8	4·8	7·2
Threonine	..	20	4·0	..	24	26·5

Poly AC (1 : 5)

Amino acid	Calculated triplet frequency				Sum of calculated triplet frequencies	Amino acid incorporation
	3A	2A1C	1A2C	3C		
Asparagine	..	3·3	..	..	3·3	5·3
Glutamine	..	3·3	..	..	3·3	5·2
Histidine	..	..	16·7	..	16·7	23·4
Lysine	0·7	..	..	..	0·7	1·0
Proline	..	..	16·7	83·3	100	100
Threonine	..	3·3	16·7	..	20	20·8

*These values are calculated from the data of Table 1. The net promotion of the incorporation of an amino acid by a certain polymer is given as per cent of the net promotion of incorporation of the amino acid whose incorporation is promoted to the greatest extent. The amino acid in question is phenylalanine for U-rich polymers, proline for C-rich polymers and lysine for A-rich polymers.

threonine each at about 20% of the lysine level. This corresponds to the frequency of each of the three 2A1C triplets relative to the frequency of the 3A (lysine) triplet. Therefore, we conclude that these three amino acids have a triplet each with the composition of 2A1C. Proline and histidine are incorporated at a level close to 4% of the lysine incorporation (Tables 1 and 2). Therefore, a 2A1C triplet each is assigned to histidine and proline. Proline also has a 3C triplet, but the frequency of the 3C triplet in the poly AC (5 : 1) (0·8% relative to that of the 3A/lysine/ triplet) is too small to account for the proline incorporation. If the triplet composition of the polymer determines the amino acid composition of the product, then a change of the base composition of the directing polymer should produce a corresponding change in the composition of the polypeptide product. Poly AC (1 : 5) (Table 2) promotes the incorporation of the same group of six amino acids as poly AC (5 : 1) discussed above. However, the incorporation ratio of these amino acids, i.e. the amino acid composition, is now altered.

TABLE 3—Data for Code Triplet Assignments (Copolymers of U and C)

Poly UC (5 : 1)

Amino acid	Calculated triplet frequency				Sum of calculated triplet frequencies	Amino acid incorporation
	3U	2U1C	1U2C	3C		
Leucine	..	16·7	3·3	..	20	22·2
Phenylalanine	83·3	16·7	..	..	100	100
Proline	..	..	3·3	0·7	4	5·1
Serine	..	16·7	3·3	..	20	23·6

Poly UC (4·7 : 5·3)

Amino acid	Calculated triplet frequency				Sum of calculated triplet frequencies	Amino acid incorporation*
	3U	2U1C	1U2C	3C		
Leucine	..	53	60	..	113	92
Phenylalanine	47	53	..	..	100	100
Proline	..	..	60	67	127	175
Serine	..	53	60	..	113	90

Poly UC (1 : 5)

Amino acid	Calculated triplet frequency				Sum of calculated triplet frequencies	Amino acid incorporation
	3U	2U1C	1U2C	3C		
Leucine	..	3·3	16·7	..	20	20·8
Phenylalanine	0·7	3·3	..	..	4	2·8
Proline	..	..	16·7	83·3	100	100
Serine	..	3·3	16·7	..	20	19·2

*See legend to Table 2.

Tables 1 and 2 show that proline is now the amino acid whose incorporation is highest, as would be expected. The incorporation is taken as 100 and that of other amino acids is expressed as per cent of proline incorporation. Proline incorporation can be divided into a portion due to the 3C triplet (83·3%) and a portion due to a 1A2C triplet (16·7%). The frequency of all the other triplets is calculated as the percentage of the sum of the frequencies of the two kinds of proline-specifying triplets. Similar considerations hold for all cases where a polymer contains more than one kind of triplet specifying the same amino acid (see Table 3). Threonine incorporation, which was about 20% of that of lysine with poly AC (5 : 1) is about 20% of that of proline with poly AC (1 : 5). This is evidence for a 1A2C triplet for threonine in addition to the 2A1C triplet assigned above, and all of the eight triplets in poly AC are thus assigned.

Copolymers containing various ratios of U and C contain eight kinds of triplets but direct the incorporation of only four amino acids (Tables 1 and 3). The ratios of incorporation are in harmony with the assignments made in Table 3 of two triplets to each of the four amino acids.

Triplet assignments

The compositions of code triplets specifying amino acids were determined in the above way utilizing random copolymers of two or three kinds of nucleotides with varying base ratios in all possible cases. Copolymers with high G content, being inactive, could not be utilized[42]. Triplet assignments based on using random copolymers, as initiated in our laboratory, have also been made by Nirenberg *et al.*[43] Most of the results from the two laboratories are in agreement. Promotion of amino acid incorporation with copolymers not containing U was first reported by Bretscher and Grunberg-Manago[31]. Table 4 lists triplets derived in *Esch. coli* so far in our laboratory. Additional data for the assignments were published in a series of communications[28]. Only the base composition of the triplets is significant in Table 4. The order of bases is only tentative and most probably wrong. The tentative assignment of order was based on (a) single amino acid replacements in protein which should be due to single base mutation[44], (b) the sequence of the 2U1A triplet for tyrosine was based on experiments with a non-random polymer (see the subsequent section for details), (c) unless there was evidence against it from (a), an attempt was made to maximize the number of "shared doublets". Thus, the sequence in triplets specifying the same amino acid and having two nucleotides in common was ordered in such a way that the two common nucleotides should occupy the same relative positions. The code is very degenerate. The majority of the amino acids are coded for by several triplets. In many but not all cases these have shared doublets. The latter are interconvertible by single base changes. Frequently this base change occurs in the same relative position of the triplets. There are several cases of changes involving A, C and U;

TABLE 4—Code Triplets

Amino acid	Code triplets†				Shared doublets‡
Alanine	CAG	CCG	CUG		C.G
Arginine	GAA	GCC	GUC		G.C*
Asparagine	CAA	CUA	UAA		C.A*, .AA*
Aspartic acid	GCA	GUA			G.A
Cysteine	GUU				..
Glutamic acid	AAG	AUG			A.G
Glutamine	AAC	UAC			.AC
Glycine	GAG	GCG	GUG		G.G
Histidine	ACC	AUC			A.C
Isoleucine	AAU	CAU	UUA		.AU*
Leucine	CCU	UAU	UGU	UUC	..
Lysine	AAA	AUA			A.A
Methionine	AGU				..
Phenylalanine	UCU	UUU			U.U
Proline	CAC	CCC	CUC		C.C
Serine	ACG	CUU	UCC		..
Threonine	ACA	CCA	UCA		.CA
Tryptophane	UGG				..
Tyrosine	ACU	AUU			A.U
Valine	UUG				..

†All triplet assignments in this list were established in the *Esch. coli* system.

‡Shared doublets that are not common for all triplets specifying the same amino acid are marked by an asterisk.

however, there is so far only one case (that of leucine) in which G takes part in such an interchange.

The physical basis for the degeneracy of the code was unravelled by separating leucine specific t-RNA of *Esch. coli* into two fractions by counter-current distribution and showing that one of these fractions transferred its leucine into polypeptide in the presence of poly UC (codon 2U1C, 1U2C), while the other responded better to poly UG (codon 2U1G)[45]. This finding indicated that there might be as many t-RNAs for an amino acid as codons assigned to it.

Table 5 shows the distribution of amino acids among code triplets. So far out of 64 possible triplets 46 have been assigned to amino acids. Some of the remaining 18 unassigned triplets might not specify amino acids, i.e. might be so-called nonsense triplets[46,47]. Recently, experimental evidence was provided for the existence of such triplets. It was shown that certain suppressible mutations (if not suppressed) produce fragments of polypeptide chains[4]. This proved that a nonsense triplet interrupts the translation beyond its location in the m-RNA.

TABLE 5—Distribution of Amino Acids Among Code Triplets

Number of triplets with identical composition	Triplets	Number of triplets assigned to amino acids	Amino acids
1	AAA	1	lys
1	CCC	1	pro
1	UUU	1	phe
3	AAC ACA CAA	3	gln thr asn
3	ACC CAC CCA	3	his pro thr
3	AAU AUA UAA	3	ile lys asn
3	AUU UAU UUA	3	tyr leu ile
3	CCU CUC UCC	3	leu pro ser
3	CUU UCU UUC	3	ser phe leu
6	ACU AUC CAU CUA UAC UCA	6	tyr his ile asn gln thr
1	GGG	0	
3	AAG AGA GAA	2	glu — arg
3	AGG GAG GGA	1	— gly —
3	CCG CGC GCC	2	ala — arg
3	CGG GCG GGC	1	— gly —
3	GGU GUG UGG	2	— gly try
3	GUU UGU UUG	3	cys leu val
6	ACG AGC CAG CGA GAC GCA	3	ser — ala — — asp
6	AGU AUG GAU GUA UAG UGA	3	met glu — asp — —
6	CGU CUG GCU GUC UCG UGC	2	— ala — arg — —

It can be seen from Table 5 that all triplets not containing G were assigned to amino acids. This suggests (a) that any copolymer which does not contain G should promote the formation of an uninterrupted peptide chain; (b) that nonsense triplets should contain at least one G.

Assigning amino acids to the residual 18 G-containing triplets is difficult because polymers with high G content are inactive.

For a discussion of the correlation between the conclusions from the study of amino acid replacements in proteins of mutants and of triplet assignments based on the study with the cell-free amino acid incorporating system, see the contribution of H. G. Wittmann in this volume, and the study of Yanofsky[48].

EFFECT OF NON-RANDOM COPOLYMERS

Studies with random polymers provided data for the assignment of the nucleotide composition of triplets. In order to obtain their nucleotide sequence non-random polymers have to be used.

Three types of synthetic non-random polynucleotides have been tested as messengers so far.

(1) Those with a unique sequence at the 3′-phosphate terminated end were made by hydrolyzing random copolymers with specific ribonucleases $(Up)_nGp$ of average chain length 40 terminated with a ^{14}C-labelled 3′-guanylic acid residue was prepared by treatment with takadiastase ribonuclease T1 which specifically hydrolyses bonds between the 3′-phosphate of guanylic acid residues and the 5′-hydroxyl of the adjacent nucleotide[49] (highly purified enzyme was kindly provided by Dr Egami and by Dr Von Ehrenstein). $(Ap)_nCp$ of average chain length 25, terminated by a ^{14}C-labelled 3′-cytidylic acid residue was prepared by treatment with pancreatic ribonuclease. $(Up)_nGp$ promoted the incorporation of phenylalanine at about 4% of the level obtained with the random polymer from which it was derived. $(Ap)_nCp$ promoted the incorporation of lysine at about 25% of the level obtained with the random copolymer from which it was derived. $(Up)_nGp$ was found not to promote the incorporation of any of the amino acids having a 1G2U triplet except leucine; similar leucine incorporation, however, is also seen with poly U. $(Ap)_nCp$ was found not to promote the incorporation of any amino acid having a 2A1C triplet. These negative results may, however, be explained by the finding that during the first two minutes of incubation up to 90% of the labelled terminal residue of the polymers was lost, probably due to exonuclease action. Thus, further purification of the cell-free amino acid incorporating system, i.e. removal or inactivation of nucleases, is required for making valid experiments with this kind of non-random copolymer.

(2) The polymer made priming poly U synthesis with ApU dinucleotide,

i.e. Ap $(Up)_n$ led to a small tyrosine incorporation as well as phenylalanine incorporation[50]. This suggests ApUpU as the sequence of the tyrosine triplet. The tyrosine incorporated was found to be partially C terminal and not N terminal. Since the C terminal amino acid is incorporated last, this suggests that the transcription of m-RNA proceeds from the right, i.e. from the 3′-hydroxyl terminated end. This assignment is tentative, and further chemical characterization of the hypothetical polyphenylalanyltyrosine is required as further evidence.

(3) Alternating AU copolymer was tested and found inactive, probably due to its secondary structure.

Recently, deoxyribopolynucleotides containing repeating di- and trinucleotide sequences, like $(TTC)_4$, $(TC)_6$, etc. were synthesized chemically[51]. These can be used as templates for enzymatic synthesis of RNA with RNA nucleotidyl transferase and might lead to the formation of polyribonucleotides which are much longer than the template[52]. Such oligoribonucleotides with repeating di- and trinucleotide sequences might be successfully utilized even with the present crude amino acid incorporating system for determining coding ratio and the sequence of nucleotides within codones. A polyribonucleotide with a repeating dinucleotide sequence might lead, if the code is a triplet code, either to the formation of a copolypeptide with an alternating sequence of two amino acids, or to a homopolypeptide.

IDENTIFICATION OF PEPTIDE PRODUCTS

Nirenberg and Matthaei identified the product into which L-phenylalanine is incorporated in the presence of poly U by the cell-free system as poly-L-phenylalanine by comparing its solubility and relative susceptibility to acid hydrolysis to that of authentic poly-L-phenylalanine[26]. The insolubility of polyphenylalanine is a handicap to chemical studies; it is possible to solubilize it by sulphonation with fuming sulphuric acid at room temperature for 8 hr.

The product into which lysine is incorporated in the presence of poly A has been identified as poly-L-lysine. The trypsin digest of this product consisted principally of di- and trilysine[35]. The trypsin digest of the product whose formation is promoted by poly AU (5 : 1) includes peptides containing lysine and isoleucine as well as others containing asparagine and lysine. This proves that synthetic copolynucleotides promote the formation of copolypeptides in the *Esch. coli* system[29].

Recently, Sanger *et al.* oxidized and subjected to partial acid hydrolysis the trichloroacetic acid precipitate of a cell-free *Esch. coli* amino acid incorporating system in which amino acid incorporation was promoted by poly UG. In the above study dipeptides of cysteic acid with phenylalanine, leucine, valine, and glycine were identified[53].

Universality of the Code

Is the code, as determined in *Esch. coli*, valid in all other species ? Some of the findings arguing for universality are:

(1) If the structural gene for alkaline phosphatase from *Esch. coli* is inserted into *Serratia marcescens* (by intergeneric episomal transfer), *Serratia marcescens* synthesizes an alkaline phosphatase that seems to be identical in several properties with the enzyme from *Esch. coli* and is different from the alkaline phosphatase of *Serratia marcescens*[54].

(2) Hemoglobin is synthesized from *Esch. coli* t-RNA bound amino acids in a cell-free system from rabbit reticulocytes[55].

(3) There is good agreement between mutational amino acid replacement data in various species and triplet assignments from the cell-free *Esch. coli* system[48–56] (see also the contribution of H. G. Wittmann in this volume).

(4) Studies with cell-free systems other than *Esch. coli* (rat liver, yeast, etc.) although dealing but with few amino acids, nevertheless show that the coding relationships in these systems are the same as in *Esch. coli*[35,57–60].

The guanine+cytosine (GC) content of the DNA from different bacterial species varies from about 25 to 75%. The extensive degeneracy of the code makes it possible for organisms of the high GC-type to utilize a triplet high in GC to specify an amino acid which may be specified by a triplet not containing GC in an organism with DNA of the low GC type (2C1U *versus* 1A2U for leucine). It could be shown nevertheless that *Alcaligenes faecalis* (DNA GC content 66%) possesses the means (activating enzyme and t-RNA) to read codons which do not contain G or C. Eighteen triplets could be assigned so far in *Alcaligenes faecalis* to the same amino acids as in *Esch. coli*[61].

The principal remaining question about the amino acid code is: why is it universal ? Whether that reflects a kind of complementarity between amino acids and sequences of nucleotides, or whether it is the result of chance and is maintained by selective pressure, has not been answered yet[62]. Cysteinyl t-RNA reduced to alanyl t-RNA or oxidized to cysteyl t-RNA donates its alanyl or cysteyl residue into protein in presence of poly UG, i.e. the polymer containing the codon which specifies cysteine and does not specify alanine[63]. This proved that there was no complementarity requirement between the code triplet and the amino acid it specifies. Whereas the isoleucyl RNA synthetase can form both valyl and isoleucyl adenylates, only the enzyme isoleucyl adenylate complex transfers isoleucine to isoleucine specific t-RNA; the same enzyme in a complex with valyl adenylate does not transfer valine to any t-RNA[64,65]. Although this suggests that the activated amino acid is involved in the selection of the t-RNA, it does not decide whether the amino acid interacts directly with a sequence of nucleotides, or acts by changing the conformation of the enzyme. Recently, it was reported that Neurospora aminoacyl t-RNA synthetases can load a chromatographic fraction of *Esch. coli* t-RNA with either phenylalanine or alanine, and that there is a competition between the two amino acids in loading (*Esch. coli* aminoacyl t-RNA synthetases do not load phenylalanine on this fraction)[66]. This ambiguity in loading (if not due to a secondary alteration of t-RNA by the heterologous Neurospora extract and thus an artifact) is an argument against a strictly amino acid specific interaction between amino acid and adaptor triplet.

Ambiguity in Translation

In the cell-free amino acid incorporating system from *Esch. coli* poly U promotes the incorporation into an acid insoluble product (in addition to phenylalanine) of a smaller and variable amount of leucine[27,30,31]. Same effect of poly U was reported in cell-free systems derived from sea urchin egg[67], chlamydomonas, and mammalian systems as well[60]. Thus, the UUU triplet behaves in an *ambiguous* fashion.

According to the adaptor hypothesis, the alignment of amino acids in protein synthesis occurs through base pairing between the individual codons in m-RNA and the complementary adaptors of the amino acid carrying t-RNA[1]. This view has received indirect support from the finding that the ability of synthetic polynucleotides containing analogs of the naturally occurring bases to promote the incorporation of amino acids into protein in the cell-free *Esch. coli* system depends on the integrity of the hydrogen bonding sites. Thus, whereas hypoxanthine can be substituted for guanine[38] and 5′-fluorouracil for uracil[36], N-3 methyluracil cannot replace uracil[36]. Hydrogen bonding through the ring nitrogen in position 3 is blocked by the methyl group in N-3 methyluracil; poly N-methyl U fails to form a twin-stranded complex with poly A[68].

If we accept pairing of codon and adaptor region as an intermediate step in amino acid incorporation, we might divide possible mechanisms by which poly U promotes the incorporation of leucine into peptides into two groups: (a) Those in which correct base pairing occurs, i.e. codon UUU pairs with adaptor region AAA. If this is the mechanism by which leucine incorporation is promoted by poly U, it must be the consequence of leucine being carried by a t-RNA with a false, i.e. phenylalanine specific, adaptor region. Thus, the error or ambiguity is in loading of t-RNA. Existence of such errors in loading of t-RNA was put forward earlier to explain suppression[69,70] (cf.[66]). (b) Those in which false base pairing occurs, i.e. the codon UUU pairs with an adaptor region consisting of, for example, two As and a third base not A.

A mechanism in which false base pairing occurs should behave in a predictable way under certain experimental conditions.

Since the complementary region is shorter in the case of false base pairing (a doublet *versus* a triplet in the correct case), it might be expected that poly U-promoted leucine incorporation will be more sensitive to changes in conditions affecting interaction of oligonucleotides with polynucleotides than incorporation of phenylalanine[71]. The stability of the 1 : 1 poly A+ poly rT complex is substantially higher than that of the 1 : 1 poly A+ poly U complex. The difference in stability is reflected in the fact that in 0·15 M sodium chloride, 0·015 M sodium citrate, the T_M of poly A+poly rT (1 : 1) is 79°, and T_M of poly A+poly U (1 : 1) is 60° (Ref. 72). It might be expected that poly rT should be a more ambiguous messenger than poly U.

Studies with synthetic polynucleotides demonstrated that temperature and Mg^{2+} concentration affect the interaction between poly- and oligonucleotides. Lowering the temperature or increasing the concentration of Mg^{2+}, is known to increase the strength of the interaction and vice versa[73]. Thus, if ambiguity was due to false base pairing, it should be increased by lowering the temperature or increasing the concentration of Mg^{2+} and vice versa.

If ambiguity in the case of leucine was due to false base pairing, it is likely that it should occur in other cases too; for example, poly U might promote a slight incorporation of amino acids which have codons containing two Us. False incorporation of these amino acids, as promoted by poly U, should behave similarly towards changes in temperature and Mg^{2+} concentration as leucine does. All these expectations were fulfilled.

Experimental results on the incorporation of phenylalanine or leucine in the presence of poly U or poly rT under different experimental conditions are shown in Tables 6 and 7 (Ref. 71). In the experiments shown in Tables 6, 7 and 8 only one-labelled amino acid was added to each incubation mixture. Cold amino acids were not added (For details, see Ref. 71). It is apparent that with greater complexing ability of the polymer there was an increase in the incorporation of leucine relative to that of phenylalanine. Thus, for a given temperature or Mg^{2+} concentration and except for the experiments with poly rT at 20°C. (Table 6) at which temperature this polymer had very little activity, the leucine/phenylalanine incorporation ratio was greater with poly rT than with poly U. The same was true of each polymer as the temperature was lowered (Table 6) or the Mg^{2+} concentration raised (Table 7). The leucine/phenylalanine incorporation ratios were highest in the experiments at 37°C. with 0·022 M Mg^{2+} and were higher with poly rT (11·2%) than with poly U (5·7%) (Table 7). Although the absolute amounts of phenylalanine and leucine incorporated per mg. of ribosomal protein varied with different batches of ribosomes, the above effects of nature of polymer, temperature and Mg^{2+} concentration on the relative incorporations of the two amino acids were always observed.

Under standard assay conditions the incorporation of ^{14}C-leucine

TABLE 6—EFFECT OF TEMPERATURE ON INCORPORATION OF PHENYLALANINE AND LEUCINE IN THE PRESENCE OF POLY U AND POLY rT*

Mg^{2+} conc. *M*	Temp. °C.	Amino acid incorporation with					
		Poly U			Poly rT		
		phe	leu	$\frac{leu}{phe}\times 100$	phe	leu	$\frac{leu}{phe}\times 100$
0·014	45	19·7	0·12	0·6	20·3	0·75	1·2
0·014	37	25·3	0·26	1·03	26·4	0·73	2·77
0·014	20	16·2	0·64	4·0	1·9	0·07	3·7

*$m\mu$moles/mg. ribosomal protein. 40 μg. polymer/mg. ribosomal protein and phenylalanine-^{14}C or leucine-^{14}C as the only amino acid added. Incubation 60 min.

TABLE 7—EFFECT OF Mg^{2+} ON INCORPORATION OF PHENYLALANINE AND LEUCINE IN THE PRESENCE OF POLY U AND POLY rT*

Mg^{2+} conc. *M*	Amino acid incorporation with					
	Poly U			Poly rT		
	phe	leu	$\frac{leu}{phe}\times 100$	phe	leu	$\frac{leu}{phe}\times 100$
0·014	24·1	0·35	1·45	25·2	0·86	3·42
0·022	21·3	1·22	5·7	16·8	2·02	12·0

*Conditions as under Table 6. Incubation at 37°C.

TABLE 8—EFFECT OF TEMPERATURE AND Mg^{2+} ON THE INCORPORATION OF PHENYLALANINE, ISOLEUCINE SERINE, AND TYROSINE IN THE PRESENCE OF POLY U*

Temp. °C.	Mg^{2+} conc. *M*	Amino acid incorporation with poly U			
		phe	ile	tyr	ser
37	0·014	22·7	0·003	0	..
20	0·014	15·1	0·106	0·014	..
37	0·014	23·5	0·002	0	..
37	0·026	15·3	0·505	0·028	..
37	0·014	23·4	..	..	0·005
20	0·014	16·3	..	..	0·030
37	0·026	14·3	..	..	0·230

*Conditions as under Table 6 with phenylalanine-^{14}C, isoleucine-^{14}C, serine-^{14}C or tyrosine-^{14}C as the only amino acid added.

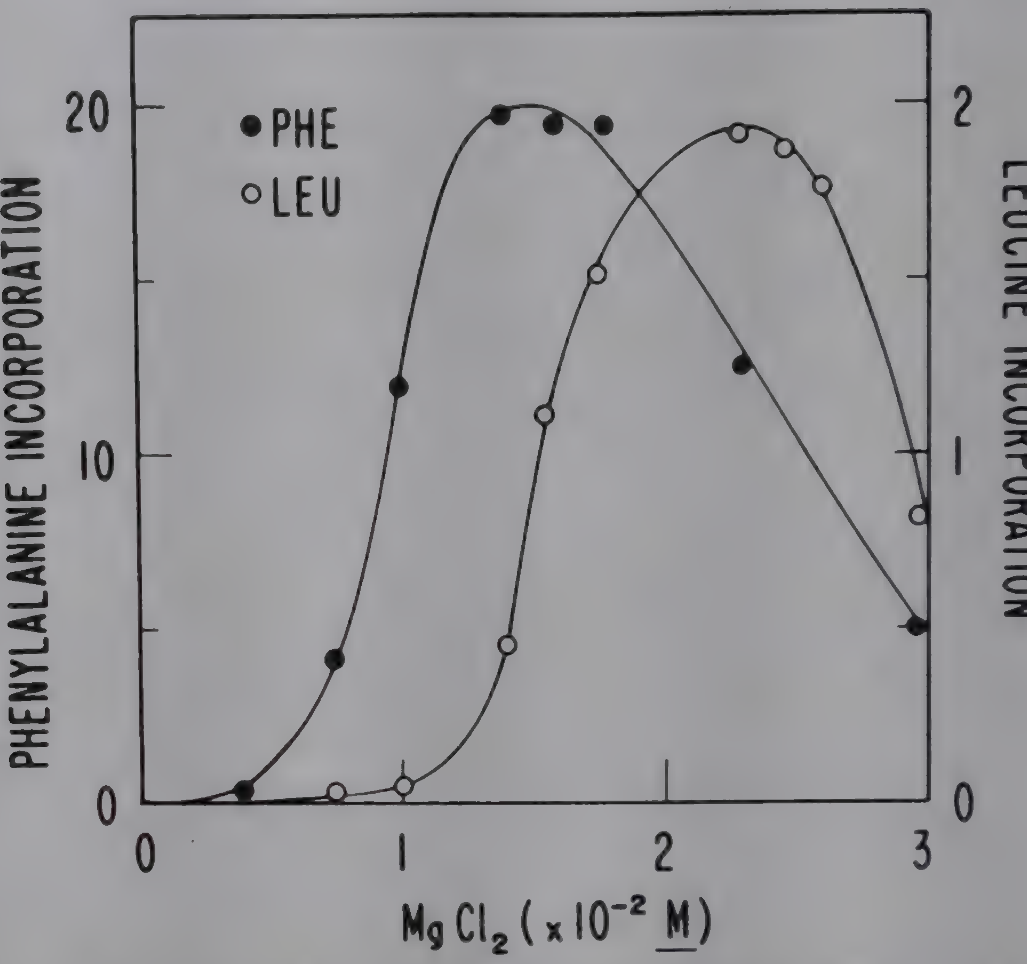

FIG. 1—INCORPORATION OF PHENYLALANINE (FULL CIRCLES) OR LEUCINE (OPEN CIRCLES), IN THE PRESENCE OF POLY U (40 μg./mg. RIBOSOMAL PROTEIN), AS A FUNCTION OF THE Mg^{2+} CONCENTRATION: Phenylalanine-^{14}C or leucine-^{14}C were the only amino acids added. Incubation 60 min. at 37°C. Amino acid incorporation expressed in mμmoles/mg. ribosomal protein

increased when the *Esch. coli* supernatant was dialysed prior to use to remove endogenous amino acids and decreased when an excess of cold phenylalanine was added to the incubation mixtures. In the presence of cold phenylalanine (0·25 μmole) and additional t-RNA the effect of poly U on the incorporation of ^{14}C-leucine was negligible. These observations suggest that phenylalanine is a strong competitor of the incorporation of leucine promoted by poly U and related polynucleotides. With poly U the incorporation of leucine also became negligible, under standard assay conditions, when the concentration of Mg^{2+} was reduced to 0·01 *M* (Fig. 1)[71]. Under these conditions the incorporation of phenylalanine was about 60% of that obtained at 0·016 *M* Mg^{2+}, which was the optimal Mg^{2+} concentration for this amino acid. As also seen in Fig. 1, the optimal Mg^{2+} concentration for leucine incorporation was about 0·022 *M*.

In the presence of poly U there was little incorporation of isoleucine or tyrosine or serine under standard conditions (37°C., 0·014 *M* Mg^{2+}), but this incorporation was significantly increased by decreasing the temperature to 20°C. or better still by increasing the Mg^{2+} to 0·026 *M* (Table 8).

There was no incorporation of lysine or valine under any of these conditions. It should be noted that conditions which led to increased incorporation of leucine, isoleucine, tyrosine, or serine, usually decreased the incorporation of phenylalanine. This fact might reflect an adverse effect of impaired polymer ribosome interaction on the incorporation of an amino acid, which, as is the case with phenylalanine, is extensively incorporated in the presence of poly U or poly rT[71]. All the above results then suggest that the ambiguity is largely due to mechanism (b), i.e. pairing mistakes between adaptors and codons.

So far four codons have been assigned to leucine. Three of these contained two Us. It might have been assumed that the high degree of ambiguity in the case of leucine incorporation with poly U was due to the existence of three two-U-containing codons. It was reported, however, that out of three leucyl t-RNA fractions separated by counter-current distribution there was one which transferred its leucine into polypeptide just as well with poly U as with copolymers with high proportions of U, i.e. those containing known codons for leucine[74]. This finding (which deserves further study under conditions known to cause less ambiguity) might indicate that the adaptor region of one of the leucyl t-RNAs is much more prone to false base pairing with the UUU triplet than others. It was reported that poly U of short chain length behaves more ambiguously than poly U with long chain[75]. Certain poly U analogs, for example, poly bromouridylic acid, promote incorporation of relatively larger amounts of isoleucine and leucine relative to phenylalanine than poly U does[76].

Streptomycin as well as kanamycin and neomycins B and C (the latter are aminoglycoside antibiotics structurally related to streptomycin) cause extensive and specific alterations in the coding properties of synthetic polynucleotides *in vitro*[77]. In addition to an inhibition of poly U promoted phenylalanine incorporation[78,79], streptomycin increases poly U promoted incorporation of isoleucine, leucine and serine, i.e. it increases the ambiguity of the system[77].

Possible Significance of Ambiguity *in vivo*

Ambiguity *in vitro* is the translation of one and the same codon into more than one amino acid. An identical phenomenon occurring *in vivo* is that of certain types of suppression. One and the same codon is translated into arginine and glycine in the tryptophane synthetase A protein in an *Esch. coli* strain containing a suppressible mutation in the cistron specifying tryptophane synthetase A and an unlinked external suppressor mutation[80]. Thus, formally, suppression might be considered as ambiguity *in vivo*. Obviously, the possible mechanisms which might account for this type of suppression can be arbitrarily divided into the same two classes which were outlined in a previous section for ambiguity.

Streptomycin on the one hand increases ambiguity in cell-free amino acid incorporating systems[77], on the other hand it suppresses certain mutations[81,82]. In a certain *Esch. coli* mutant grown in the absence of streptomycin, ornithin transcarbamylase activity was practically absent (it behaved like an arginine auxotroph); in the same strain grown in the presence of streptomycin, there was ornithin transcarbamylase activity at about 0·2% level of that in the wild type—it behaved like a prototroph[81]. The property of streptomycin to promote misreadings in translation might explain its *in vivo* suppressing effect[77]. Thus, in the structural cistron of this enzyme in the mutant strain there was a codon altered by the mutation which specified incorporation of amino acid B (in place of amino acid A in the wild type) and caused the formation of inactive enzyme. Streptomycin increased the probability of misreading; it led to an occasional incorporation of amino acid A (or another amino acid leading to a partially or fully active enzyme) by misreading the mutated codon which in reality specified amino acid B. This explanation of the mode of the suppressing action of streptomycin is similar to one originally proposed as a general mechanism for the action of external suppressors, the difference being that in the original hypothesis it was proposed that the misreading was the consequence of loading an amino acid on a t-RNA with a codon specifying a different amino acid[69,70]. The dual effect of streptomycin causing ambiguity *in vitro* and suppression *in vivo* supports the idea of the relatedness of ambiguity and suppression. It suggests that certain cases of suppression are due to false base pairing, the mechanism proposed to account for ambiguity. This proposal is reinforced by the fact that streptomycin affects the ribosomes (see later) in causing both ambiguity and suppression. Thus, it seems that ambiguity is not an artifact of the cell-free system but a reflection of a property of the protein synthesizing system existing *in vivo*.

It was reported in several communications that certain enzymes are more thermosensitive if extracted from thermophilic bacteria grown far below their normal temperature of growth than if extracted from the same bacteria grown at higher temperature[83–85]. No explanation was proposed for these findings. A further careful study of this phenomenon is obviously warranted to rule out trivial explanations. It is possible that the difference in thermosensitivity might be the result of the increased ambiguity of the protein synthesizing system working far below the normal temperature to which it is adapted and giving rise to proteins with occasional amino acid replacements. A support for this interpretation comes from the observation that the cell-free extract of a thermophile grown at high temperature is more ambiguous at low temperature than at high temperature, behaving the same way as the *Esch. coli* system does, and that at low temperature it shows errors in addition to those shown by the *Esch. coli* system[86]. An alternative explanation of the above phenomenon, which cannot be ruled out at this time, is false folding of proteins at an inappropriate temperature.

Other cases in which ambiguity *in vivo* might play a role are some of the thermosensitive mutants[87]. Presumably, in some of these it is not the protein formed that is heat-labile, but its synthesis. It might be assumed that, if in order to obtain an active protein, misreading of a missense or nonsense codon is required, this might preferentially occur at low temperature.

Involvement of Ribosomes in Translational Ambiguity

A postulate that the site of action of streptomycin is on the ribosomes[88] was experimentally verified by demonstrating that in the *in vitro* system poly U-promoted phenylalanine incorporation was inhibited only if the ribosomes were taken from sensitive cells[78,79]. It did not matter whether the supernatant originated from sensitive or resistant cells. Recently, this site of action was further narrowed to the 30s ribosome[89,90] and it was shown that the ambiguity increasing effect of streptomycin was also restricted to ribosomes of streptomycin sensitive cells[77]. Therefore, Davies, Gilbert and Gorini proposed that ribosomes may have a role in suppression. It was pointed out earlier that one has to assume that the interaction between codon and adaptor must be reinforced by some other component. This component, which might be the 30s ribosome[91] might contribute to the specificity of the interaction. A modification in this component might permit binding of not quite complementary codon and adaptor and thus lead to ambiguity.

Can ribosomes then contribute to ambiguity even if it is not due to streptomycin ? Experiments show indeed that damaging ribosomes by preincubation at high temperature leads to an increased *in vitro* ambiguity with poly U (Lengyel–Erdos, unpublished). This latter finding reinforces the possibility that changing Mg^{2+} concentration or temperature might contribute to changing the level of ambiguity by affecting the conformation of the ribosomes themselves as well[77], in addition to changing the strength of interaction between codon and adaptor.

Ambiguity and Triplet Assignments

In the light of the ambiguity of the system, do the triplet assignments from the *in vitro* system become questionable ? The experiments on which the assignments were based were performed under conditions of low ambiguity, i.e. high concentration of t-RNA and low concentration of Mg^{2+}. It seems to us that most of the assignments, particularly those which are based on experiments with several copolymers containing the same kinds of nucleotides in widely different proportions, are safe. If there are errors in the assignments at all, most probably they will affect triplets which were assigned on the basis of very low amino acid incorporations.

The high levels of ambiguity (up to 50%) reported in *Esch. coli* B[31,76,92] are due to conditions of preparing and testing the system. The study of the variables affecting the levels of ambiguity led last but not least to the elucidation of the conditions under which ambiguity is minimal.

SUMMARY

The use of homopolynucleotides and copolynucleotides of random base sequence as messengers in a cell-free amino acid incorporating system derived from *Esch. coli* led to the deciphering of the nucleotide composition of 46 triplets specifying amino acids. Utilizing random copolynucleotides containing the same kinds of bases in widely varying proportions it was verified that the base composition of the messenger determines the amino acid composition of the peptide product. Evidence was provided that the product formed with synthetic polynucleotide templates is of peptide nature. All experiments so far support the universality of the code, but the cause of its universality is only a matter of speculation.

Poly U exhibits an ambiguity in coding. It promotes incorporation of smaller amounts of leucine besides phenylalanine. The ambiguity becomes more pronounced with conditions that increase the complexing ability of the polymer. It is higher with poly rT than with poly U, and increases in either case with decreased temperature or increased Mg^{2+} concentration factors that increase the interaction between oligo- and polynucleotides. Incorporation of other amino acids having a codon containing two Us and a third non-U nucleotide is promoted by poly U also, but at an even lower level than that of leucine. Under conditions of low Mg^{2+} concentration, high concentration of t-RNA and phenylalanine, leucine incorporation is not promoted by poly U. All this points at pairing of not quite complementary codon and adaptor as the cause of ambiguity. Analogy of ambiguity *in vitro* and suppression *in vivo* is discussed.

ABBREVIATIONS USED

DNA, deoxyribonucleic acid; RNA, ribonucleic acid; m-RNA, messenger ribonucleic acid; t-RNA, transfer ribonucleic acid; GTP, guanosine 5′-triphosphate; ATP, adenosine 5′-triphosphate; A, C, G, U, the bases adenine, cytosine, guanine, uracil, also the corresponding nucleoside or nucleotide residues in polynucleotides; poly A, polyadenylic acid; poly C, polycytidylic acid; poly G, polyguanylic acid; poly I, polyinosinic acid; poly rT, polyribothymidylic acid; poly AC, polyadenylic-cytidylic acid; poly UC, polyuridylic-cytidylic acid; in the shorthand writing of polynucleotides, as in $(Up)_nGp$, the latter to the right of the nucleotide initial indicates a 3′-phosphate; the amino acids are abbreviated by the first three letters of their names; except isoleucine, ile; asparagine, asn; glutamine, gln.

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The Specific Partial Hydrolysis of Nucleic Acids after Chemical Modification

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It has been possible within the past few years, to obtain information about the nature of the genetic code with indirect methods (Nirenberg, Lengyel, Wittmann, Yanofsky, Crick). A direct comparison of nucleotide sequences in nucleic acids with the corresponding amino acid sequences in polypeptide chains is, however, desirable. The essential feature of the adaptor hypothesis of Crick and Hoagland, the complementary binding between nucleotide triplets in messenger and amino acid transfer RNA (s-RNA), has not been proven.

For these and other reasons, the sequence analysis of nucleic acids is of interest. The major problems to be solved are: (1) The isolation of single molecular species of nucleic acids. This problem has been solved for viral nucleic acids, which may be obtained from purified virus particles, and, to a certain extent, for amino acid specific s-RNA. The separation of the complex mixture of nucleic acids, as it is extracted from cells, is still not possible. (2) The specific fragmentation or the defined stepwise degradation of nucleic acids. (3) The recognition of the fragments obtained by specific partial hydrolysis of nucleic acids. The separation of small oligonucleotides is possible today. The separation of sequence isomers of oligonucleotides and of larger nucleic acid fragments has not been worked out.

The following report deals only with the second problem. Since nucleic acids, except the s-RNA, are specific irregular sequences of a large number of nucleotide residues belonging to only 4 different types, it would be necessary for sequence determinations to have methods available which yield large fragments. Even with 4 independent methods splitting specifically at the four different types of nucleotide residues, the problem cannot be solved. It is, however, desirable to design techniques which yield, specifically, large nucleotide fragments.

For s-RNA, the solution of the problem is much simpler, (i) because they contain only 60 to 100 nucleotide residues per chain and (ii) because

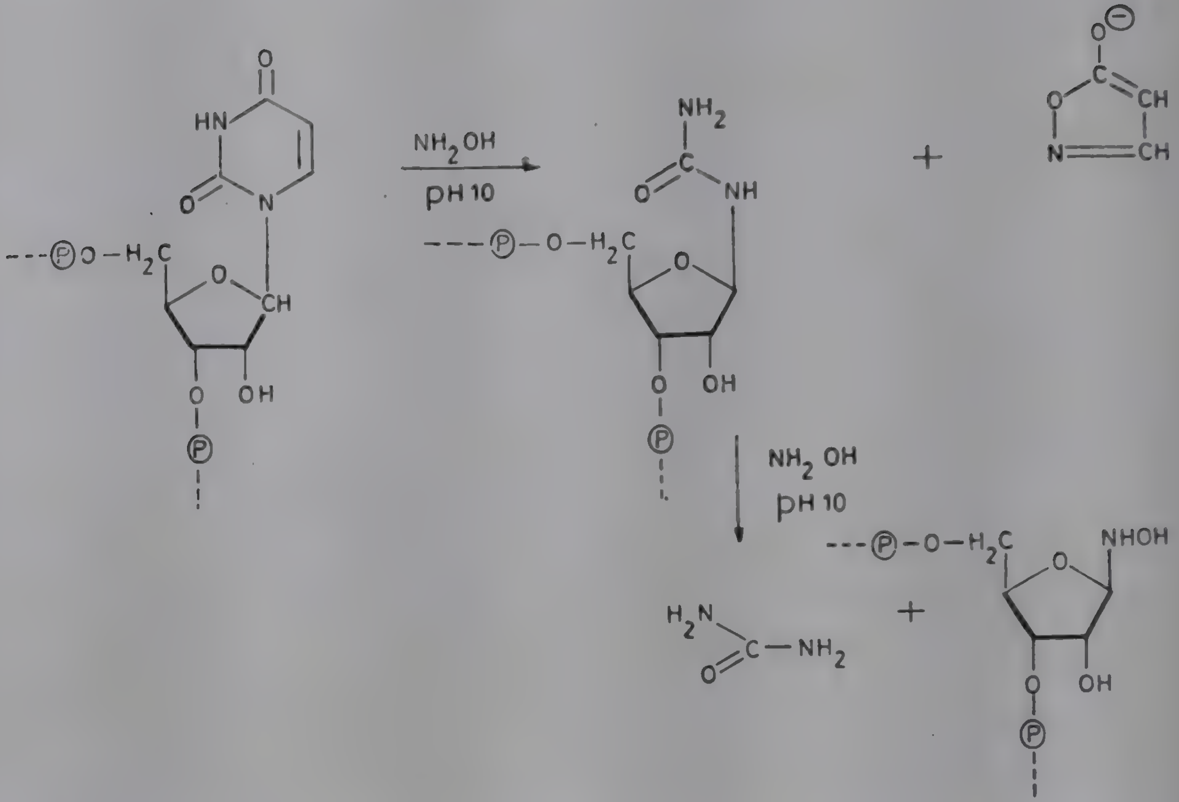

FIG. 1—REACTION OF HYDROXYLAMINE WITH URIDINE AND URIDYLIC ACID IN s-RNA

of the presence of pseudouridylic acid and a number of methylated nucleotide residues. When 4 independent hydrolysis procedures specific for the 4 normal nucleotide residues do not suffice, it should be possible to devise methods for a specific split besides one of these abnormal nucleotides.

The only well introduced method giving rise to one specific split is the use of takadiastase-ribonuclease I, which hydrolyses besides guanylic acid residues (Egami). Pancreatic RNAase which splits besides both normal pyrimidine nucleotides, would be a valuable tool for sequence analysis if it should be possible to confine its action to only one of the two pyrimidine nucleotide species. The diester bonds of two of the abnormal nucleotides present in s-RNA, pseudouridylic acid (pseudo-Up or ψ Up) and ribothymidylic acid (rTp) which amount to 2-4 residues per chain in the average, are attacked by pancreatic RNAase. It should be possible, after elimination or modification of the normal pyrimidine bases, to obtain specific hydrolysis at these sites.

A specific modification of one of the normal pyrimidine bases is possible with hydroxylamine[1,2].

In RNA, at *p*H 10·0, in 6 *M* aqueous hydroxylamine solution within 3 hr practically all the uracil residues are eliminated, whereas only 7·5% of the cytosine residues have been modified. Pseudouridylic acid and ribothymidylic acid residues remain unchanged. The product is a desuracil-RNA in which uracil residues have been replaced by hydroxylamino groups. The 3-carbon fragment of uracil is bound to hydroxylamine giving rise to free isoxazolon-5 (Fig. 1).

At *p*H 6·0, with 6 *M* aqueous hydroxylamine the cytosine residues of RNA are quantitatively modified within several hours without a significant reaction of uracil residues. The product of the reaction is an RNA, which, at this *p*H, contains instead of cytosine, 4, 5-dihydro-5-hydroxylamino-uracil-6-oxim moieties.

At acid *p*H, the hydroxylamino residue bound to carbon 5 is eliminated, so that uracil-6-oxim residues are formed. Again, the abnormal pyrimidines do not react (Fig. 2).

The *p*H-dependence of the specificity of the reaction is shown in Fig. 3.

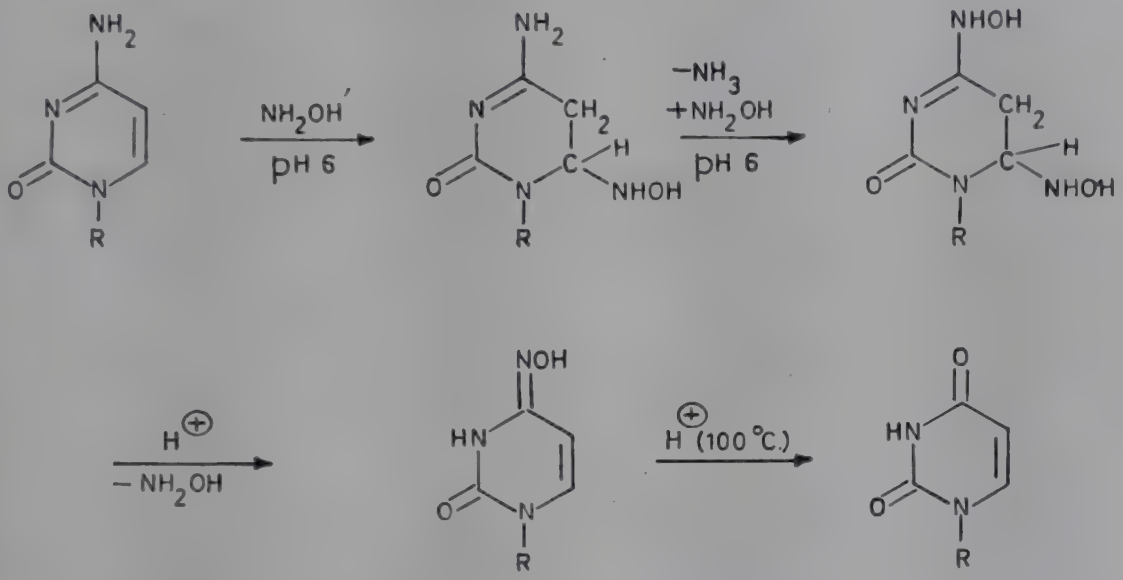

FIG. 2—REACTIONS OF CYTOSINE AND CYTIDYLIC ACID WITH HYDROXYLAMINE

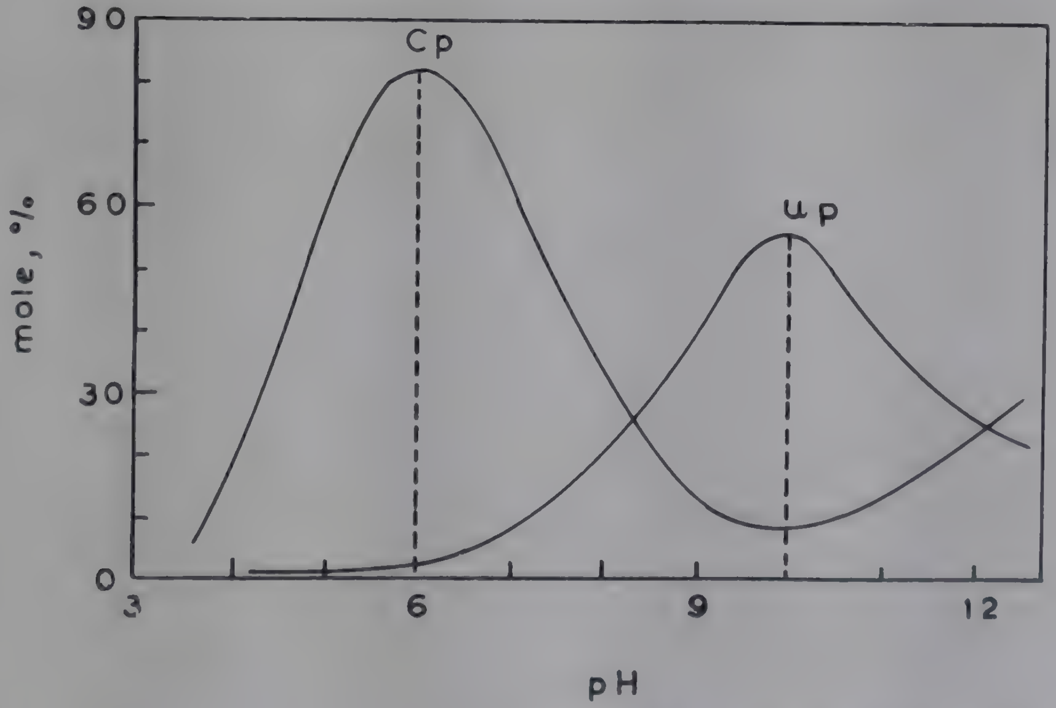

FIG. 3—*p*H-DEPENDENCE OF THE RATE OF REACTION OF 6 *M* AQUEOUS HYDROXYLAMINE WITH CYTIDYLIC ACID AND URIDYLIC ACID

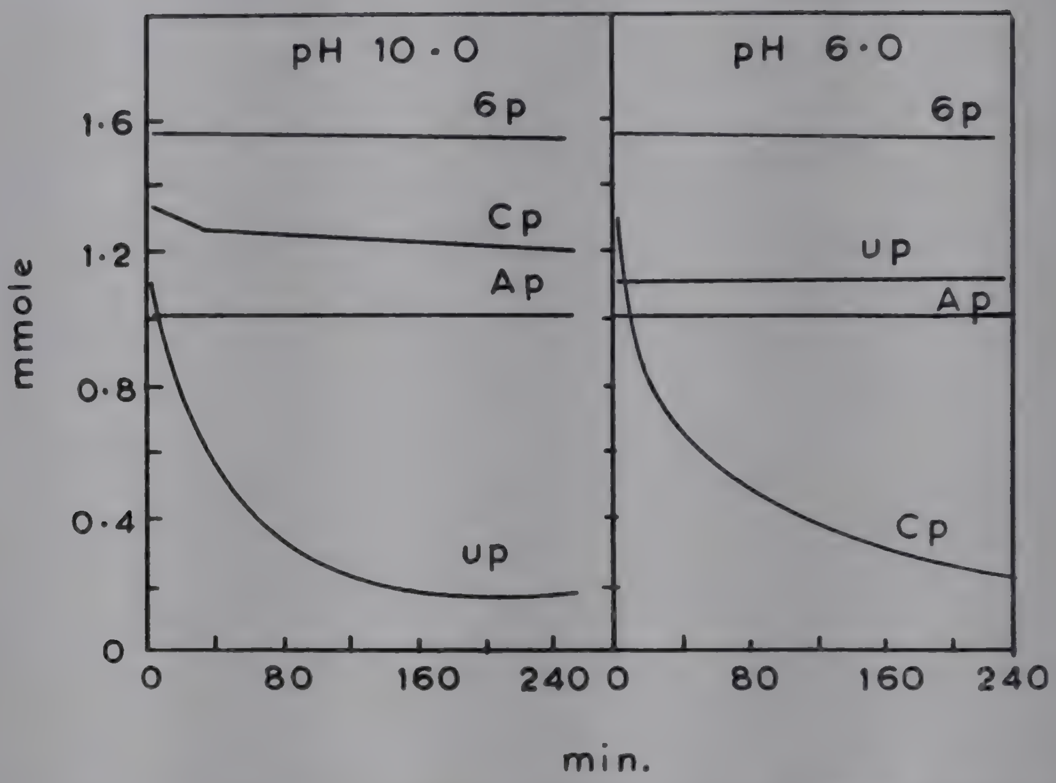

FIG. 4—RATES OF REACTION OF MONONUCLEOTIDE COMPONENTS OF s-RNA TREATED WITH 6 *M* HYDROXYLAMINE AT 37°C.

The kinetics of the reactions of yeast s-RNA with hydroxylamine at *p*H 10 and *p*H 6 are presented in Fig. 4.

The product of the *p*H 10-reaction of s-RNA is attacked by pancreatic RNAase specifically at the cytidylic acid-, pseudouridylic- and ribothymidylic acid sites. No split is observed at the ribosyl-hydroxylamine sites originating from uracil elimination. The modified cytidylic acid sites are still attacked by pancreatic RNAase. Thus a C- (and pseudo U- and ribo-T) specific split is obtained. In the resulting oligonucleotides, the former uracil residues can still be recognized as ribosylhydroxylamino moieties.

The evidence for these conclusions has been published[3]. The random hydrolysis during the time of the reaction of the RNA with hydroxylamine does not exceed 1 split per chain and is of the same order as that occurring in the absence of hydroxylamine. Since this is a statistical event, the result of the analysis of the hydrolysis product will not be seriously changed.

The product of the *p*H 6-reaction of s-RNA is still attacked by pancreatic RNAase at the sites where C has been modified as well as at the sites of the other pyrimidine nucleotides. Therefore, a complementary U-specific split cannot be obtained in this way[3].

A specific alkaline split of the product of the reaction of RNA with hydroxylamine at *p*H 10 appears to be possible by β-elimination or after further chemical modification. It has, however, not been possible to obtain evidence for an increased alkaline lability of the primary reaction product. When the hydroxylamino residues are removed by treatment with 5 *M* glyoxylic acid at *p*H 4·5, the RNA contains instead of uridylic acid residues free ribose moieties, which should allow β-elimination of the adjacent phosphodiester bonds in analogy to the Whitfeld-degradation. When such an RNA, after treatment with glyoxylic acid, is reacted with periodate, the ribose residues are transformed into tetrose moieties, which, again should have labelled adjacent phosphodiester bonds. Hydrolysis of s-RNA, treated in this way, can indeed be demonstrated by high voltage electrophoresis on Sephadex-G 75-blocks. Further investigation will be required to decide if these or similar methods will be of practical value.

Since the abnormal pyrimidinenucleotides, pseudo-Up and ribo-Tp, present in s-RNA, do not react with hydroxylamine, it appeared possible after elimination of U with hydroxylamine at *p*H 10 and of C with anhydrous hydrazine, to obtain, with pancreatic RNAase, a pseudo-U and ribo-T specific split of s-RNA. As hydroxylamine, hydrazine does not attack pseudo-U. Ribo-T is reacting very slowly. When a yeast s-RNA, treated successively with hydroxylamine and hydrazine, is incubated with pancreatic RNAase, the alkali consumption corresponds to the amount of newly formed phosphomonoester groups which should result from a specific split at the sites of pseudo-U and ribo-T[4].

By chromatography on DEAE cellulose according to Tener (6 *M* urea, ammonium bicarbonate gradient *p*H 8·6) it has been demonstrated that only a small amount of fragments with chain lengths below 6 was present.

The specificity of the RNAase-hydrolysis can be proved by (i) treating the hydrolysis product with diesterase free prostata phosphomonoesterase and (ii) hydrolysis of the resulting oligonucleotide mixture. The monoesterase removes monoesterphosphate from the endgroups so that the alkaline hydrolysis yields, besides nucleotides, nucleosides derived from the endgroups, originally terminating with 3′-phosphate.

The nucleosides can be separated from the nucleotides by chromatography on Dowex 1 × 8 and identified by high voltage electrophoresis and paper chromatography. Besides pseudo-U and ribo-T and adenosine originating from the s-RNA endgroup excess adenosine and guanosine have been found. This, probably comes from random hydrolysis during the various steps of the procedure. It should be possible to decrease the amount of undesired side products by using highly purified enzymes and by better control of the chemical steps of the procedure.

The value of this method depends on the possibility to distinguish in the fragments obtained after RNAase hydrolysis the positions at which originally U and C were bound. The ribosyl hydroxylamine residues which are introduced in the first reaction steps are partially substituted by ribosehydrazone moieties during the following treatment with anhydrous hydrazine.

Therefore, it was necessary to modify the ribosyl hydroxylamine residues in such a way that they would be resistant against hydrazine.

Catalytic reduction to ribitylamin residues which was possible when free ribosyl hydroxylamine was used as a model, did not succeed under mild conditions with hydroxylamine treated s-RNA. After removal of the hydroxylamino groups with 1 *M* glyoxylic acid at *p*H 4·5, the free ribose residues in s-RNA, can be reduced by 0·5 *M* sodium boranate at *p*H 8·0-9·5 within 1 hr. The resulting ribitol groups are resistant to hydrazine treatment. Therefore, in the fragments obtained by hydrolysis with pancreatic RNAase, an unambiquous recognition of the sites to which U and C had been bound, is possible (Fig. 5).

In summary, after removal of U from s-RNA with hydroxylamine at *p*H 10, pancreatic RNAase splits specifically at the sites of C, pseudo-U and ribo-T.

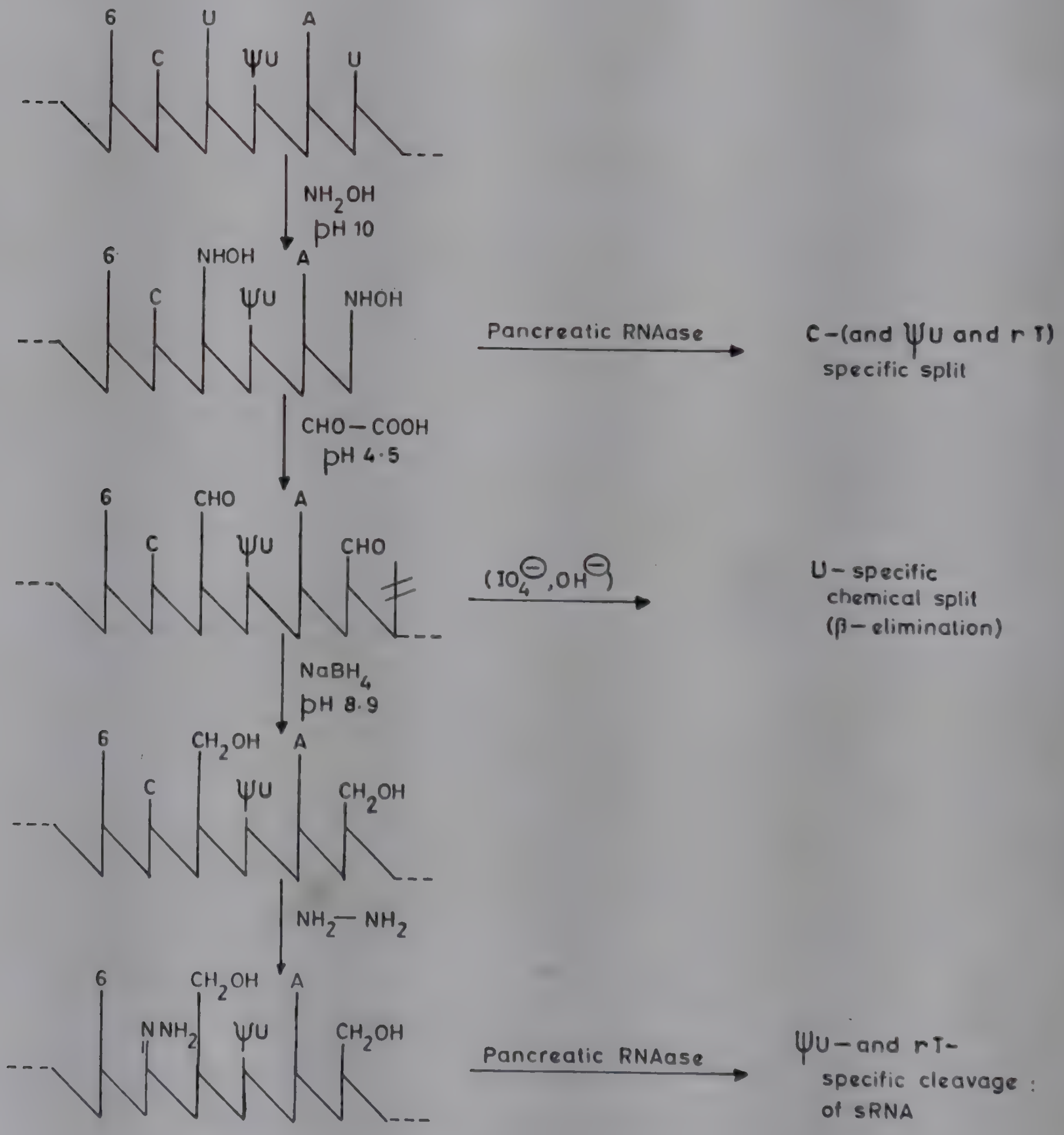
6
U
A
C
ψU
U
NH2OH
pH 10
6
NHOH
A
C
ψU
NHOH
Pancreatic RNAase
C-(and ψU and r T)
specific split
CHO—COOH
pH 4·5
6
CHO
A
C
ψU
CHO
(IO4⊖,OH⊖)
U-specific
chemical split
(β-elimination)
NaBH4
pH 8·9
6
CH2OH
A
C
ψU
CH2OH
NH2—NH2
6
CH2OH
A
NNH2
ψU
CH2OH
Pancreatic RNAase
ψU-and rT-
specific cleavage :
of sRNA

FIG. 5—METHODS FOR SPECIFIC DEGRADATION OF s-RNA

A chemical hydrolysis at the sites, to which, originally, U was bound, appears to be possible.

After removal of U by hydroxylamine and of C with hydrazine, specific cleavage at the sites of pseudo-U and ribo-T can be obtained.

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Origin of Nucleic Acids

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The cell is an hierarchic system in which certain species of macromolecules control the production and function of other macromolecules. At the top of the system stands the deoxyribonucleic acid (DNA) of the genes in which the information necessary for the functioning and reproduction of the entire system is stored (Fig. 1). This predominant role of nucleic acids in biology is evident from the development of a virus. In the first moment after infection of a cell only one molecule of viral nucleic acid, either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) is present. This molecule induces the formation of a number of enzyme proteins which catalyse the formation of more viral nucleic acid of the same type. Finally the multiplied virus nucleic acid induces the formation of the proteins which encapsulate the nucleic acids to form complete new virus particles. This course of events was carefully studied by Hausen[1] for the ME (Maus-Elberfeld) virus, which belongs to the entero group, and by Epstein *et al.*[2] for the DNA containing bacteriophages.

It seems probable then, that one of the first steps in the development of life is the formation of a nucleic acid containing the information for its own reduplication. Living organisms are systems of an outstanding improbability. For instance, the probability for the spontaneous formation of the nucleic acid of tobacco mosaic virus (TMV) is approximately 10^{-2000} as determined from simple statistical consideration of the number of ways 4 different nucleotides can be arranged in chains of 6000 units. Even if the total cosmos, the mass of which has been estimated to be 10^{60} g. were composed entirely of nucleotides, and these nucleotides reacted with the velocity of fastest reactions known in chemistry for a period of time equal to the age of the cosmos, estimated to be of the order of 10^{9} years, no more than 10^{100} bonds between two nucleotides could be formed, a number too small for an event of a probability of 10^{-2000} to be realized by chance. What is required is a process of evolution involving multiplication, mutation, and selection through which the information for the construction of organisms is gradually accumulated. So far as we know this information can only be accumulated in nucleic acids and not with proteins and the

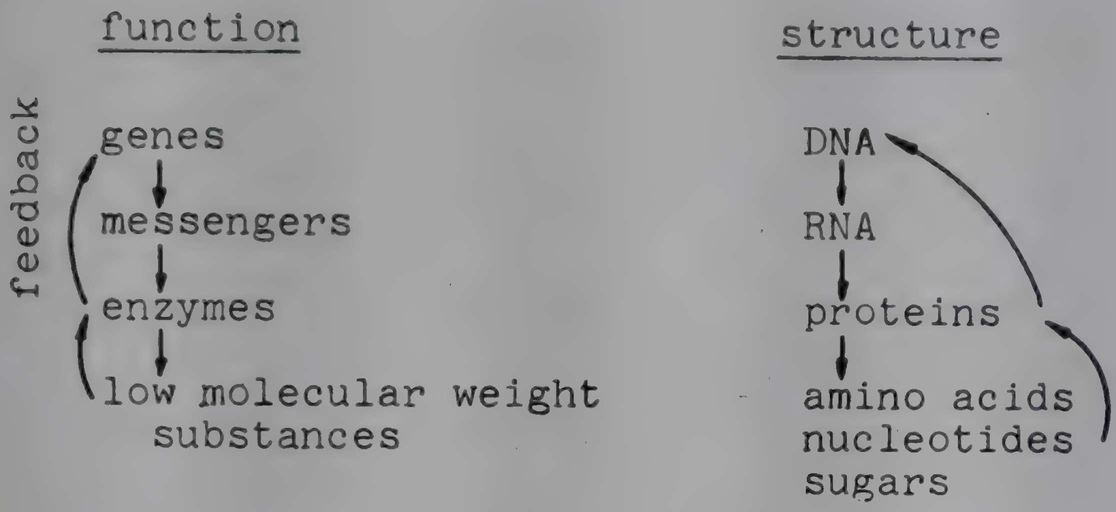


FIG. 1—CELL ORGANIZATION

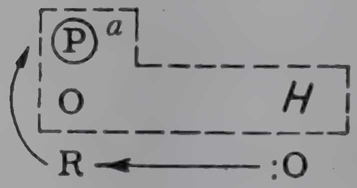
other constituents of the cell. The problem of the origin of life is, therefore, equivalent to the problem of the origin of nucleic acids.

In the living organisms of to-day the synthesis of nucleic acids is achieved by a series of reactions involving the elimination of an hydroxy group from one molecule and a proton from another. The energy necessary for these syntheses is provided by adenosine triphosphate (ATP) which converts inert nucleoside monophosphates to reactive nucleoside diphosphates or nucleoside triphosphates. In effect the hydroxy group of the phosphate is substituted by a phosphate or pyrophosphate group. By this substitution the electrons are withdrawn from the α-phosphorus atom bound to the nucleoside and the resulting electron deficiency can be filled by the OH group of the second nucleotide. This mechanism is used by nature in the synthesis of many biopolymers (Table 1). Instead of phosphorus a carbon atom can also be attacked by nucleophilic substituents such as OH, NH, SH, or CH.

The activating phosphoryl group should not contain a negative charge close to the central atom since this would repel the electrons. In the cell the negative charges of the phosphoryl or pyrophosphoryl groups are probably removed by protonization or by a condensing enzyme. In non-enzymatic reactions the simplest way to remove the charges is the esterification of the phosphates. We found in our laboratory that many of these biological condensations can be imitated by use of a simple cyclic metaphosphate ester (MPE), the structure of which will be described later. With this agent we achieved the formation of polypeptides from amino acids, polyglycosides from simple sugars, nucleosides from sugars and bases and finally the condensation of nucleotides to polynucleotides[3].

Although all of these macromolecules have important functions in living organisms, I will confine myself to the non-enzymatic synthesis of nucleic acids. Nucleic acids are originated from sugars and nitrogen

TABLE 1—Biosyntheses by Elimination of Water: α=phosphate-, pyrophosphate- or adenylate residue. R=glycosyl-, carboxyl-, isopentenyl- or nucleotidyl residue

Condensation	Product
	Polyglycoside; R = C Polynucleotide; R = P Aminoacyl RNA ⟶ Polypeptides
$\text{Ⓟ}OR \longleftarrow :NH$	Nucleosides
$\text{Ⓟ}OR \longleftarrow :SH$	Acetyl-CoA ⟶ Fatty acids
$\text{Ⓟ}OR \longleftarrow :CH$	Polyisoprene R = Isopentenyl

bases by 3 subsequent condensation steps: first between sugar and base leading to nucleosides; second between nucleosides and phosphoric acid leading to nucleotides; and third between one nucleotide and another one leading stepwise to long polynucleotide chains. All the three types of condensation can be achieved by the metaphosphate ester. For the origin of nucleic acids the following preconditions are required: (i) the presence of pentoses, (ii) the presence of purines and pyrimidines, (iii) the presence of metaphosphate ester. It is possible that these conditions are met in the primordial atmosphere of the earth. It is known[4] that the early atmosphere contained simple combinations of the 4 elements C, N, H, and O. The experiments of Calvin *et al.*[5], and Miller[6] have shown that in fact with sufficient energy a great variety of organic compounds can be made, including the essential building blocks of proteins and nucleic acids. We can consider now in more detail possible steps in the formation of nucleic acids.

Formation of sugars

Already Butlerow and Loew (1861) and Emil Fischer demonstrated that sugars can be obtained from formaldehyde in alkaline solution. Recent studies[7,8] have shown that the reaction is rather specific. In the presence of hydroxides and carbonates of the alkaline earth metals mainly pentoses

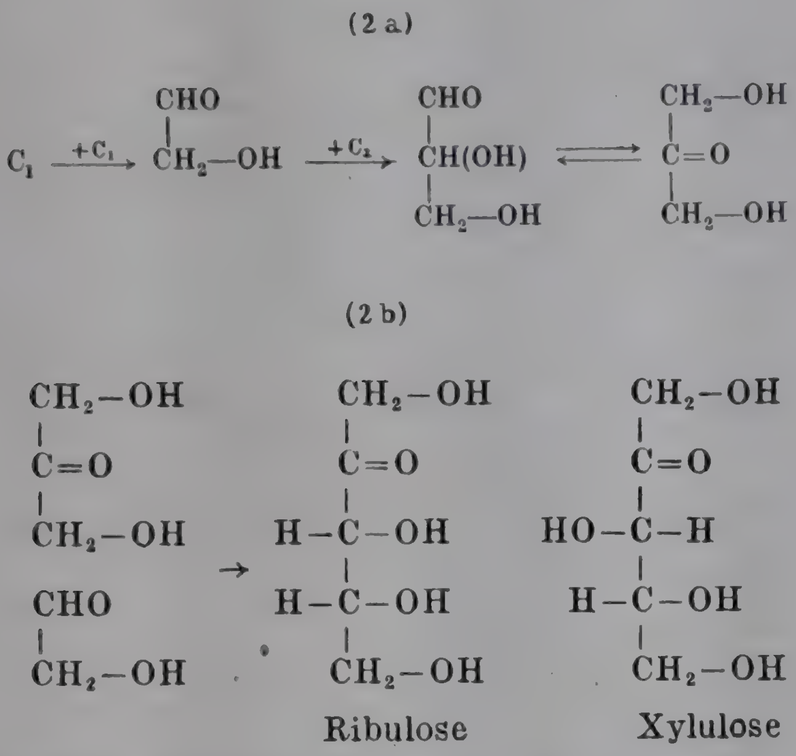


Fig. 2—(a) Condensation of 3 moles of formaldehyde to glyceraldehyde and isomerization to dihydroxyacetone. (b) Condensation of dihydroxyacetone with glycolaldehyde to pentoses

and especially ribulose are formed. A few hexoses were also detected. The reaction mechanism can be seen from Fig. 2a and 2b. By condensation of 3 moles of formaldehyde, glyceraldehyde is formed, but it is converted rapidly to dihydroxyacetone. This reacts slowly with formaldehyde, but very rapidly with glycolaldehyde to yield ribulose. Oró[9] showed that 2-deoxypentose can be formed easily in aqueous solution from glyceraldehyde and acetaldehyde. This study shows that large amounts of pentoses and deoxypentoses could have been available in primordial conditions.

Formation of purines and pyrimidines

Oró[9] and his associates have also shown that purines and pyrimidines can be generated under very simple conditions. Adenine can easily be formed by the polymerization of hydrogen cyanide in aqueous ammonia. Intermediates were isolated and he proposed a detailed mechanism for the synthesis of purines under primitive conditions which is shown in Fig. 3. Uracil and other pyrimidines can be formed by the condensation of 3-C compounds such as acrylonitrile or aminopropioamide in aqueous ammonia at 135°C.

Formation of metaphosphate ester

Metaphosphate esters can be prepared in the laboratory in different ways. One of the simplest methods is the reaction of 2 moles of ethyl-ether with phosphorus pentoxide (P_4O_{10}) as studied in our laboratory[10]. Below

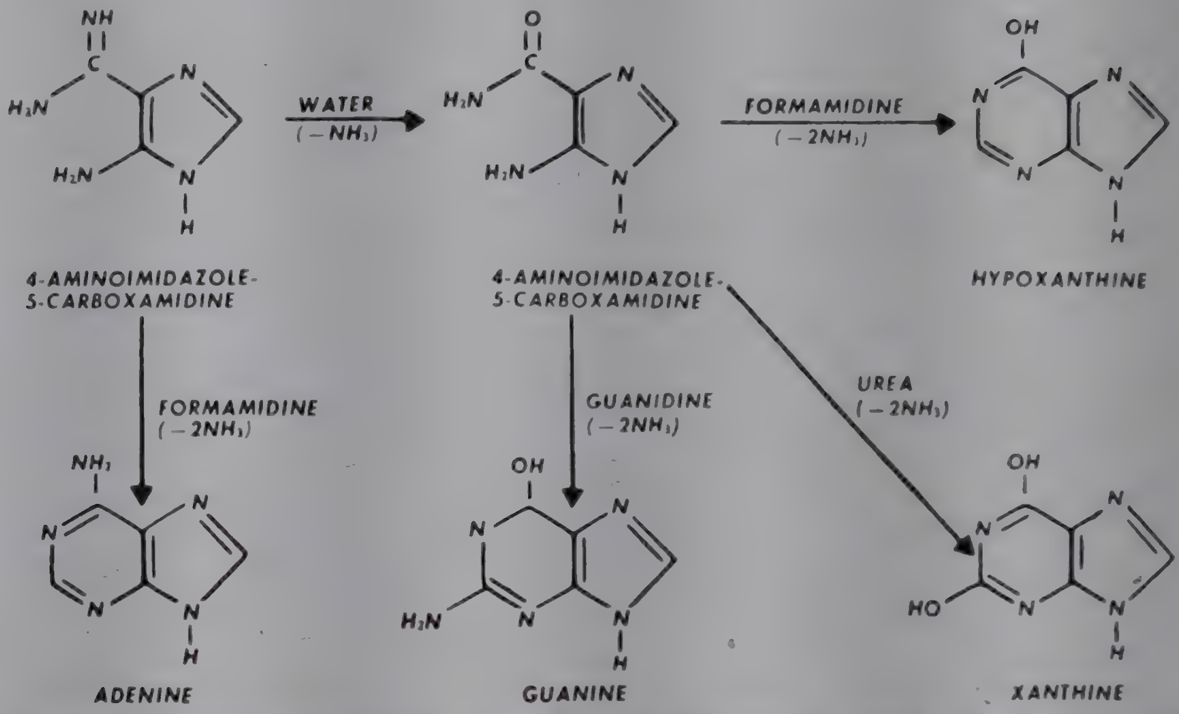

4-AMINOIMIDAZOLE-
5-CARBOXAMIDINE
WATER
(−NH₃)
4-AMINOIMIDAZOLE-
5-CARBOXAMIDINE
FORMAMIDINE
(−2NH₃)
HYPOXANTHINE
FORMAMIDINE
(−2NH₃)
GUANIDINE
(−2NH₃)
UREA
(−2NH₃)
ADENINE
GUANINE
XANTHINE

FIG. 3—MECHANISM PROPOSED BY ORÓ[9], FOR THE SYNTHESES OF PURINES ON THE PRIMITIVE EARTH

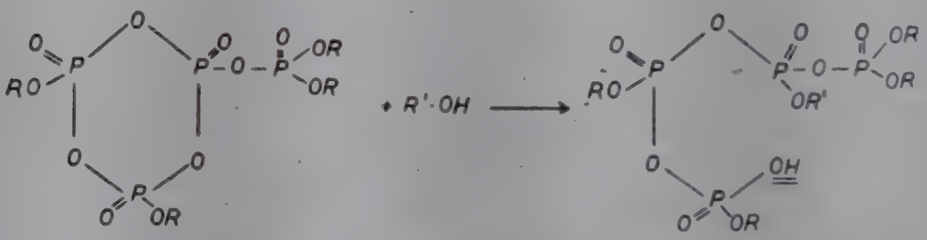


FIG. 4—REACTION OF ISO-TETRAETHYLMETAPHOSPHATE ESTER WITH HYDROXY COMPOUNDS: R′=alkyl-, glycosyl-, carboxyl- or phosphoryl residue

60°C. only 2 moles of ether react with P_4O_{10} and a mixture of tetrametaphosphate and isotetrametaphosphate ethyl ester (Fig. 4) is formed. The structure of these compounds was already correctly described by Rätz and Thilo[10] and confirmed recently by Pollmann and Schramm[11] and by Weill *et al.*[12] It is quite possible that metaphosphates were formed under the conditions present in the primordial earth. Orthophosphates are not stable above 300°C., but large water-free deposits of condensed phosphates presumably developed as the earth cooled. By reaction of a variety of organic compounds this material could be esterified, thus producing metaphosphate esters in a water-free environment to catalyse the condensation reactions mentioned above. Oparin[13] has suggested that the water-free space for these reactions might be within droplets protected by a water-proof membrane similar to coacervates.

Formation of nucleosides

We have indicated that the required condition for nucleic acid synthesis could have been present during the early history of the earth, and can now

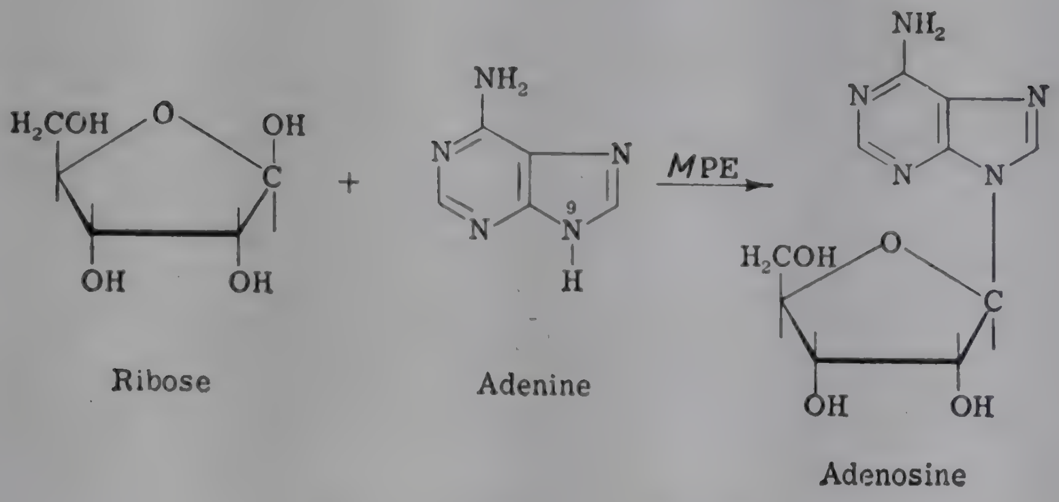


FIG. 5—FORMATION OF ADENOSINE

discuss possible reactions leading to the polynucleotides. Nucleosides are formed by the condensation of ribose and another sugar with a heterocyclic base, a combination which can easily be effected by metaphosphate ester. The cyclic phosphate ester reacts very rapidly with hydroxy and amino compounds. Only one P-O-P bond is split and an esterified tetrametaphosphate residue is attached to the hydroxy or amino group (Fig. 4). The kinetics can be followed by titration in non-aqueous solution[10]. At 50°C. ribose combines with 2 moles of MPE and by experiments with other sugars we found that the acetalic OH on C_1 is the most reactive group and next reactive is the primary OH on C_5. The secondary OH groups on C_2 and C_3 do not react under the conditions used in our experiments. The simplest assumption would be that the nitrogen replaces the substituted OH group of the sugar directly, but the reaction is complicated in that the NH groups in the bases also react with MPE. By subsequent concerted reaction both activating phosphate ester groups are removed and the C-N bond shown in Fig. 5 is formed.

From ribose and adenine only the natural β-glycoside configuration was obtained. This is due to the directing influence of the OH group at C_2. With deoxyribose, in which this OH group is missing, a mixture of the α- and β-nucleosides was formed. The great advantage of this procedure is that sugars and bases can be used directly without protecting groups. In spite of the simplicity of the reaction the yields of adenosine and deoxyadenosine are better than with the conventional methods using protecting groups. It is, therefore, quite possible that this type of nucleoside formation has occurred in the primordial history of the earth.

Formation of polynucleotides

Condensation between the phosphate groups of a nucleotide and the hydroxy group in the pentose of another nucleotide proceeds by a mechanism analogous to the condensation of a sugar with a base. By titration in non-aqueous solution it can be demonstrated that the phosphate group as well as the hydroxy group in the other nucleotide reacts with one mole of MPE. Subsequently during the combination both activating residues are eliminated. This reaction is less dependent on the proton concentration than in the case of the nucleosides. It proceeds in the presence of tertiary bases, an important factor in applications to nucleotides unstable in acidic medium such as deoxyadenylic acid or deoxyguanylic acid.

In order to obtain polynucleotides of high molecular weight the concentration of nucleotides should be as high as possible, the best condition being the application of MPE without any additional solvent. It is necessary to have homogeneous reaction conditions which cannot be obtained without using special precaution. By freeze drying an aqueous solution of nucleotides under a good vacuum a very finely divided preparation is obtained which can be dissolved easily in MPE. A homogeneous solution can also be obtained by ultrasonic vibrations with rigorous cooling. Alternatively one can add a small amount of phosphoric acid tris dimethylamide which reduces the viscosity of MPE and enhances the dissolution of the nucleotides. Other solvents are inhibitory. Humidity should be carefully excluded.

The polymerization reaction produces a mixture of condensates with a broad distribution of molecular weights, which can be characterized and fractionated by column chromatography on Sephadex G 25, G 50, and G 75 or on DEAE cellulose. The high molecular weight fractions can be further characterized in the ultracentrifuge and molecular weights higher than 10,000 have been recorded. In the top fractions long filaments of polynucleotides have been observed in the electron microscope but it has to be ascertained whether these filaments consist of a single polynucleotide chain or of aggregates of smaller molecules. For the purification of the polynucleotides the oligonucleotides and part of the condensing agent can be removed by dialysis and the remaining MPE can be separated from the polynucleotides by fractionation on DEAE cellulose. Products have been obtained which have a P content close to the theoretical value.

In the natural polynucleotides the phosphate links the C-3′ position with the C-5′ position. In the synthetic products besides these natural linkages other phosphate bonds are possible. The polymerization of thymidylic acid led to a high molecular weight product which contained a considerable amount of 5′-5′-pyrophosphate linkages, easily detectable by degradation with acetic anhydride in pyridine. The polyribonucleotides, as far as they have been studied, are stable against this agent and contain no detectable amount of pyrophosphate linkages as expected due to the interaction with adjacent OH group. Polyuridylic acid, obtained by polymerization from the commercial mixture of 2′- and 3′-uridine monophosphate is not fully degraded by ribonuclease. This indicates the presence of unnatural linkages. The degradation with snake venom phosphodiesterase, which is specific for phosphate in 5′ position, is more complete.

This indicates that most of the wrong linkages are of the 2′-5′ type. If both hydroxy groups in 2′ and 3′ are substituted the phosphate linkage to the adjacent nucleotide will become stable against alkaline hydrolysis. Therefore by alkaline hydrolysis some information can be obtained about the number of branching points. The polymerization of nucleotides with MPE was studied recently by Kochetkov *et al.*[14] The molecular weight was estimated to be 10000, although no exact measurements were made. From enzymatic and alkaline hydrolysis the authors concluded that most linkages are unnatural. These results differ from those obtained in our laboratory. One reason for this may be that the starting material used by the Russian authors contained a large amount of 2′-nucleotides that are resistant to ribonuclease.

Matthaei, H. (personal communication) compared the coding activity of our synthetic poly-uridylic acid with that of poly-uridylic acid prepared using phosphorylase, the incorporation of phenylalanine having been determined in a cell-free system of *Escherichia coli.* Since smaller polynucleotides are inactive or even inhibitory, a fractionation of the preparations was necessary. Fractions of the synthetic polyuridylic acid and the enzymatic polyuridylic acid with the same molecular weight had approximately the same coding activity. Therefore, the structure of the synthetic product is apparently not too different from that of the natural product, although further experiments will be made to try to improve the specificity of the polymerization.

Formation of informational polynucleotides

In conclusion, our experiments have shown that a great variety of macromolecules can be obtained by means of metaphosphate esters. However, these polymerizations lead to only random arrangements of the monomers in the macromolecules and the specificity of the linkages is not as high as in natural polymers, as one would certainly expect from probability considerations. We are faced then with the problem of how large information bearing molecules with specific sequences and linkages are formed non-enzymatically, or in other words how to induce specificity into our polymerization reactions.

In nature the specificity of polymer formation is controlled by a template mechanism. The prototype is the multiplication of DNA in which one template controls the formation of the complementary one and this reversibly determines the structure of the original strand. On the basis of this model we tried to influence the polymerization process by the addition of templates. We found that the polymerization of uridylic acid to polyuridylic acid is not catalysed by the end product polyuridylic acid, but only by the complementary polyadenylic acid. In other words polyadenylic acid contains the information necessary for building polyuridylic acid. Since on the other hand polyuridylic acid can induce the formation of

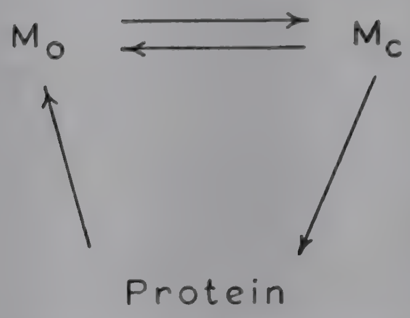


Fig. 6—Scheme of a self-organizing system : M_0=original matrix. M_c=complementary matrix

polyadenylic acid, storage and transmission of information is possible. The template which is multiplied at the highest rate will replace the less suitable ones, and so a selection according to the ability of multiplication can be expected.

The DNA of the organisms contains not only the information for its own multiplication, but also for the construction of enzymes and other proteins. These enzymes control the multiplication of DNA directly or indirectly via the metabolism of the total organism. This is illustrated in the simplified scheme shown in Fig. 6, which demonstrates the simplest form of a self-regulating or self-organizing system. One catalyst controls the formation of the other one and thereby its own formation. It would be interesting to know whether such a system can be verified by simple chemical components. We found that the formation of polyuridylic acid can be accelerated by the addition of simple basic polypeptides formed with MPE, but we have not studied whether a synthetic nucleic acid can catalyse the formation of a polypeptide. It may be that this occurs by simple amino acyl polynucleotides which can easily be formed in our prebiological mixtures of condensed material. I am quite aware that this scheme is hypothetical, but I think it has some heuristic value in stimulating new experiments.

Polymerization of triplets

It was found that oligonucleotides, including trinucleotides, can also be condensed by MPE to higher polymers. This may have some bearing for the solution of the genetic code. The experiments of Crick *et al.*[15] make it very probable that the genetic code consists of triplets. The composition of these triplets could be elucidated by use of random polynucleotides for incorporation of amino acids in a cell-free system[16-19]. Further information was obtained by tobacco mosaic virus mutation experiments of Wittmann[20]. But both methods give no information about the sequence of the nucleotides in the triplet. If during our polymerization with MPE no rearrangement and splitting occur in the triplets, the polymerization with MPE should lead to specific nucleotides which code for only one amino acid. By combination of all 64 triplets the function of the complete triplet code could be analysed.

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Some Recent Experiments on the Role of Histones as Regulators of Chromosomal Activity

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There is now much evidence that in the differentiated cells of higher organisms many genes are inactive. Genes that are active in one cell type may be inactive in other cell types and in a given cell type the activity of genes may vary considerably in response to changes in the environment.

The experiments to be described are concerned with the role of histones in nuclear function, with particular emphasis on biosynthetic reactions which modify the structure of histones by the introduction of acetyl and methyl groups. These reactions have been studied in isolated calf thymus nuclei *in vitro*, using acetate-2-^{14}C and methionine-methyl-^{14}C (Ref. 1) as precursors, comparing their incorporation with that of ^{14}C-lysine and other amino acids, and testing the effects of puromycin on the synthesis of different histone fractions. Evidence will be presented to show that in the cell nucleus, the acetylation and methylation of the histones occur very probably after completion of the polypeptide chain.

Such modifications of histone structure, acetylation in particular, may affect the capacity of the histones to inhibit ribonucleic acid synthesis *in vivo*. This view is supported by the finding that, when isolated arginine-rich histones are subjected to a limited acetylation, they lose much of their effectiveness as inhibitors of RNA synthesis by the DNA-dependent RNA polymerases of calf thymus nuclei or *Escherichia coli*. Yet such modified histones are still strongly basic proteins which retain an affinity for DNA comparable to that of the parent histone from which they were derived.

The findings introduce the possibility that histone effects on nuclear RNA metabolism may involve more than a simple inhibition of RNA synthesis, and that more subtle mechanisms may exist which permit both inhibition and reactivation of RNA production at different loci along the chromosome.

Interest in the histones as regulators of chromosomal activity has heightened considerably in the past few years, as increasing experimental evidence has accumulated to support the view that the role of histones is to inhibit chromosomal function in differentiated cells in higher animals and plants. The inhibitory role of histones was originally surmised by Stedman and Stedman[2] in 1951, but biochemical evidence that histones do block DNA function is of recent origin. Of particular interest are the observations (*a*) that histones inhibit RNA synthesis in isolated thymus nuclei[3-5], (*b*) that removal of the histones from the nucleus results in an increased rate of "messenger" RNA polymerase from pea-seedling nuclei[7]. The inhibition of RNA polymerases by histones has been observed in mammalian[6,8] and bacterial systems as well[9-11], although it should be pointed out that non-specific polycations, such as polylysine, will also inhibit RNA polymerase activity[11], and that histones can inhibit a wide range of other enzymatic reactions not directly involved in RNA synthesis[6,12-14].

It has seemed likely that the suppression of RNA synthesis by histones is due in large part to complex formation between the added histones and the DNA needed as a "primer" in the RNA polymerase reaction. Indeed, the correlation between DNA binding and the inhibition of RNA synthesis is evident in the experiments of Huang and Bonner[7], who showed that histones which protected DNA from thermal denaturation also blocked its "primer" action.

Something more, however, than simply the combination of histone with DNA should be considered. The experiments to be described below indicate that combination without inhibition is possible, and they suggest that specific (and presumably reversible) changes in histone structure, which do not prevent binding of histone to DNA, may permit DNA to serve as a template in the RNA polymerase reaction. This raises the possibility that relatively minor modification of histone structure, taking place on the intact protein molecule, offers a means of switching-on or -off RNA synthesis at different loci along the chromosome.

Histone synthesis in isolated cell nuclei

Amino acid incorporation into histones. It has been shown previously that nuclei isolated from calf thymus tissue in isotonic sucrose solutions can incorporate amino acids into their proteins[15,16]. The histones become labelled in the process[16], although they are not as rapidly synthesized as are other protein fractions of the nucleus[15] (Fig. 1A). There are good reasons to believe that the synthesis or "turnover" of histones is intranuclear[16] and that it can proceed without a concomitant synthesis of DNA, because thymus lymphocytes are not dividing rapidly, and because histone "turnover"

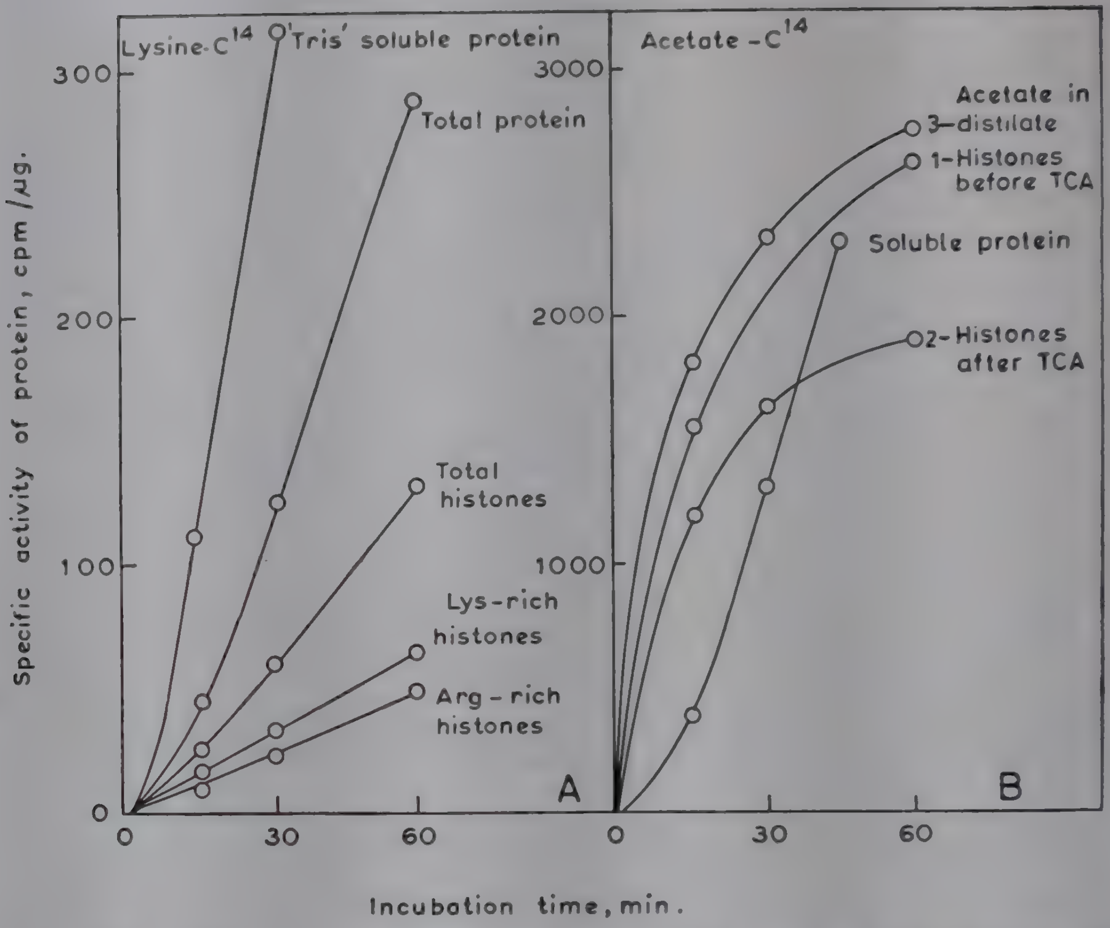

Fig. 1—Time course of lysine-1-^{14}C (A) and acetate-2-^{14}C (B) incorporation into the proteins of isolated calf thymus nuclei: The total histones were prepared by acid extraction of nuclei previously washed with 0·01 *M* Tris buffer (*p*H 7·6) to remove soluble proteins and ribosomes. The lysine-rich and arginine-rich histones were prepared by chromatography on carboxymethylcellulose. Curve 3 in (B) gives the total counts recoverable in the distillate from 1 mg. of hydrolysed histone

has been observed in non-dividing tissues of adult animals[17]. Amino acid incorporation into the histones, or into other proteins of isolated thymus nuclei, is an energy-dependent process. It is inhibited by agents which block nuclear ATP synthesis[14,18], but it is not affected by Co^{2+}, Ca^{2+} ions, or methylene blue, all of which block mitochondrial ATP synthesis without affecting nuclear ATP levels[14]. The synthesis of the histones like that of other nuclear proteins, appears to require a prior and continuing synthesis of RNA[8,15]. This is one reason for the lag phase in amino acid uptake (Fig. 1A). Agents which block nuclear RNA synthesis, such as actinomycin D[8,19] or 5, 6-dichloro-β D-ribofuranosyl benzimidazole[15,20], have no immediate effect on histone synthesis, but the rate of histone labelling begins to fall off 30-60 min. after the cessation of "messenger" RNA synthesis. The synthesis of thymus "messenger" RNA and its isolation are described elsewhere[5,21].

Inhibition of histone synthesis by puromycin

The incorporation of amino acids into the histones is inhibited by the addition of puromycin to the incubation medium. This antibiotic, widely

TABLE 1—EFFECTS OF PUROMYCIN ON ^{14}C-AMINO ACID INCORPORATION INTO HISTONES AND OTHER PROTEINS OF ISOLATED NUCLEI

Protein tested	Puromycin concentration μg./ml.	Specific activity of proteins after 60 min. incubation with					
		Lysine-1-^{14}C		Arginine-G-^{14}C		Alanine-1-^{14}C	
		c.p.m./mg.	% inhibition	c.p.m./mg.	% inhibition	c.p.m./mg.	% inhibition
Total nuclear protein	0	275	..	370	..	158	..
	100	28·1	89·8	50·8	86·3	21·2	86·6
Total histones	0	95·1	..				
	100	36·7	61·4				
Lysine-rich histones*	0	142	..			55·1	..
	100	75·5	53·1			24·4	44·3
Lysine-rich histones†	0	136	..				
	100	69·1	50·8				
Intermediate lys-rich histones*	0	42·6	..				
	100	10·9	74·4				
Arginine-rich histones*	0	44·5	..				
	100	11·0	75·3				
Arginine-rich histones‡	0	156·3	..	114·3	..		
	100	27·4	82·5	32·2	71·8		

*Histones prepared by chromatography on carboxymethylcellulose columns[28].
†Histones prepared by direct extraction in 5% perchloric acid[29].
‡Histones extracted in 80% ethanol–1·25 *N* HCl[29].

used as an inhibitor of protein synthesis since its introduction for this purpose by Yarmolinsky and de la Haba[22], effectively stops nuclear protein synthesis at concentrations of 100 μg./ml.[23] Since its action involves an interference with transfer-RNA function[22] and leads to a release of unfinished peptides from ribosomal sites of synthesis[24], it can be concluded that histones, like other nuclear proteins, are probably made on nuclear ribosomes[25-27].

When the synthesis of different types of histones is compared (Table 1), some are found to be less susceptible to puromycin inhibition than others. The uptake of amino acids into the lysine-rich histones, in particular, seems more resistant to this level of the antibiotic than is amino acid uptake into the arginine-rich fraction (Table 1), but the inhibition is still definite.

Modification of histone structure

Acetylation of histones in isolated nuclei. The presence of acetyl end-groups in thymus histones was detected originally by Phillips[30], who recently observed that 54-58% of the total N-terminal groups are acetylated[31]. Histones differ in this respect; the lysine-rich histones are highly

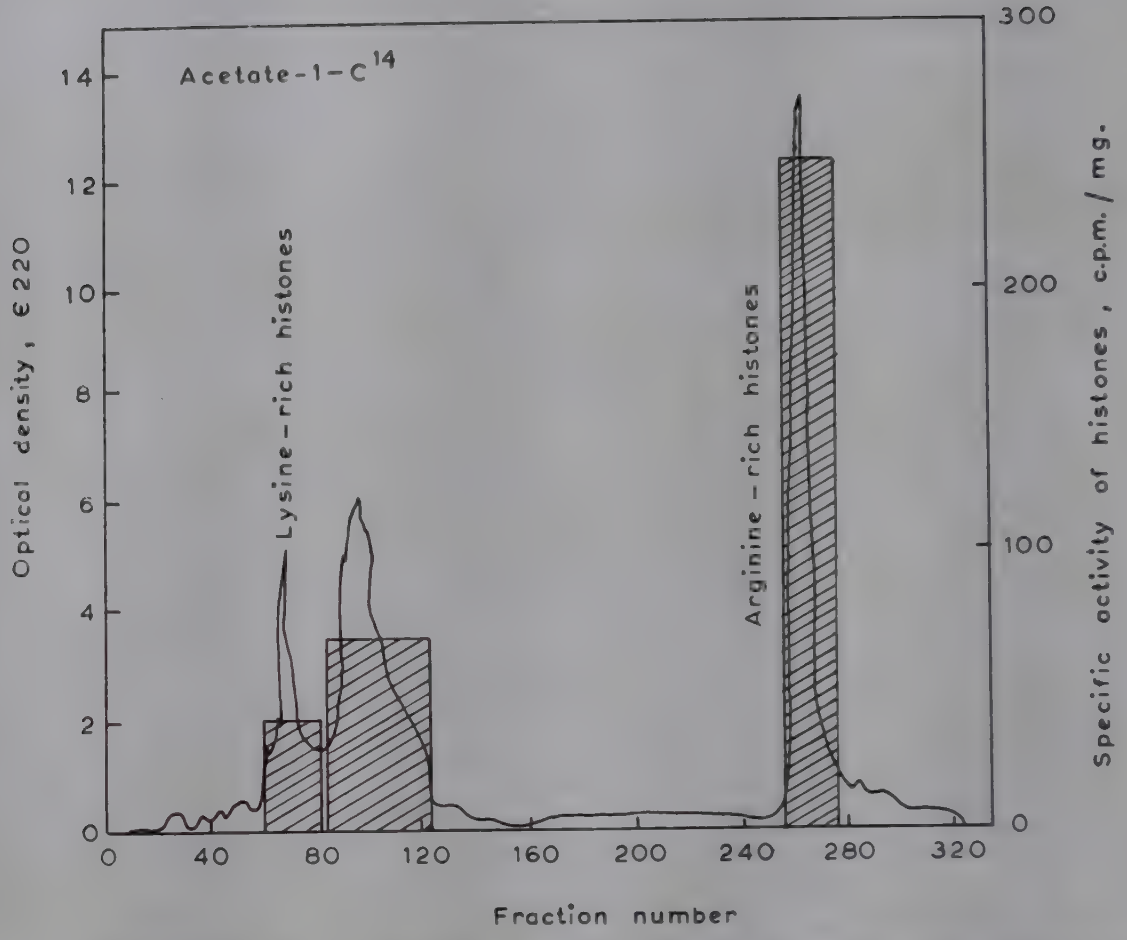

FIG. 2—SEPARATION OF ^{14}C-ACETYL-LABELLED HISTONES ON CARBOXYMETHYLCELLULOSE (CMC) COLUMNS: Nuclei were incubated 60 min. with sodium acetate-2-^{14}C and the total histone fraction was adsorbed to CMC. Elution was carried out using a salt gradient at *p*H 5·0, followed by 0·1 *N* HCl. The specific activity of the different histones is shown by the height of the cross-hatched areas

acetylated (73-80% N-terminal acetyl groups) as compared with 36-48% N-terminal acetyl groups in the arginine-rich fraction[31].

We have observed that when isolated nuclei are incubated in the presence of sodium acetate-2-^{14}C there is a rapid incorporation of isotope into the histones. The time course of uptake into histones and other proteins is shown in Fig. 1B. It is of interest that acetate-^{14}C uptake into the histones proceeds without a lag phase and tapers off quickly, while the uptake of lysine-^{14}C into the basic proteins shows the typical lag and proceeds longer (Fig. 1A). In this respect, acetate uptake into histones resembles the uptake of uridine and other precursors into nuclear RNA[32,33].

Evidence that the acetate taken up is incorporated as acetyl groups attached to histones has been obtained in several ways: (a) purification of the histones by chromatography shows that the label is eluted with the basic proteins (Fig. 2); (b) acetyl-labelled histones can be separated by electrophoresis on cellulose polyacetate strips[34]; (c) acetate-^{14}C is not removed when the histones are treated with organic solvents (to remove lipids); and (d) more than 75% of the acetate-^{14}C remains after the histones are treated with hot 16% trichloroacetic acid (TCA) at 90°C. for 15 min. (to remove polysaccharides and nucleic acids).

When TCA-treated histones are hydrolysed to their constituent amino acids (using 6N H_3PO_4 at 110°C. for 20 hr), the ^{14}C-acetyl is released as ^{14}C-acetic acid. This can be separated from the amino acids by steam distillation, and trapped in dilute alkali. Scintillation counting of the distillate allows a measure of the ^{14}C-acetyl content of the histone proteins. These counts are plotted in Fig. 1B, curve 3. Two-thirds of the total counts are recoverable as acetate-^{14}C.

Comparison of acetate-^{14}C uptakes into different histone fractions

When the acetyl-labelled histones are separated by chromatography on carboxymethyl-cellulose columns[28], it is observed that different types of histone differ in their rates of ^{14}C-acetate uptake. The specific activity of the arginine-rich fraction is particularly high (Fig. 2). This is a surprising finding considering the relatively low total acetyl content of this fraction as compared with that of lysine-rich histones[31].

Because of the extensive acetate uptake into the arginine-rich histones, this fraction was selected for studies of the effects of acetylation on histone inhibition of the RNA polymerase reaction (see below).

Failure of puromycin to inhibit acetate uptake into histones. Although puromycin effectively inhibits the uptake of arginine-G-^{14}C, lysine-1-^{14}C, and other amino acids into histones (Table 1), it has no effect on the uptake of acetate-2-^{14}C into the basic proteins of the nucleus. The

TABLE 2—COMPARATIVE EFFECTS OF PUROMYCIN ON ACETYLATION OF HISTONES AND AMINO ACID INCORPORATION INTO NUCLEAR PROTEINS

Conditions of experiment	Isotopic precursor	Specific activity of nuclear proteins				Activity recovered as ^{14}C-acetate from histones‡ c.p.m.
		Total protein c.p.m./mg.	Soluble proteins* c.p.m./mg.	Histones† c.p.m./mg.	Residue c.p.m./mg.	
Control	Lysine-1-^{14}C	487	1203	215	611	..
Nuclei + 100 μg. puromycin	Lysine-1-^{14}C	65·1	102	57·9	38·4	..
Control	Acetate-2-^{14}C	1229	1343	2100	849	2724
Nuclei + 100 μg. puromycin	Acetate-2-^{14}C	857	253	2076	381	2688

*Proteins plus ribosomes extractable in 0·01 M tris buffer (pH 7·6).
†Histone specific activity after extraction with 16% TCA at 90° for 15 min.
‡Acetate recovered by steam distillation of histone hydrolysate (see text). More than 70% of total counts in histones after acetate-labelling experiments are recoverable as acetyl-^{14}C.

differences between acetylation and synthesis are clear-cut when one compares the effects of puromycin on lysine-l-^{14}C and acetate-2-^{14}C uptakes (Table 2). The table lists the specific activities of the histones in c.p.m./mg. and also gives the total counts recovered as acetate-^{14}C from the basic protein hydrolysates.

The failure of puromycin to inhibit the acetylation reaction indicates that histones are very probably modified by acetylation after completion of the polypeptide chain. It is, therefore, unlikely that acetate is activated and transferred by sRNAs as are the amino acids. This conclusion is in accord with recent work on the acetylation of tobacco mosaic virus protein, in which no evidence for acetyl-amino acid intermediates could be obtained[35,36].

Methylation of histones in isolated nuclei

The presence of ϵ-N-methyl-lysine in calf thymus histones was recently detected by Murray[1], who also observed that the group donor for histone methylation was methionine[1].

We have studied the incorporation of ^{14}C-methyl groups into the histones of isolated thymus nuclei by incubation in the presence of L-methionine-methyl-^{14}C, and tested the effects of puromycin on this process. The results are presented in Table 3. It is clear that concentrations of puromycin which inhibit synthesis of the non-histone proteins by over 90% and which lowered lysine-^{14}C uptake into the histones by 73% had no effect on methylation of the histones. Thus, it is very probable that methylation of the histones, like that of amino acid-transfer RNAs[37,38] proceeds after the synthesis of the primary structure of the molecule has been completed.

TABLE 3—COMPARATIVE EFFECTS OF PUROMYCIN ON METHYLATION OF HISTONES AND AMINO ACID INCORPORATION INTO NUCLEAR PROTEINS

Conditions of experiment	Isotopic precursor	Specific activity of nuclear proteins				Activity recovered as methyl-lysine in histones† c.p.m.
		Total protein c.p.m./mg.	Soluble proteins* c.p.m./mg.	Histones c.p.m./mg.	Residue c.p.m./mg.	
Control	Lysine-1-^{14}C	487	1203	215	611	..
Nuclei + 100 μg. puromycin	Lysine-1-^{14}C	65·1	102	57·9	38·4	..
Control	Methionine-M-^{14}C	725	1301	198	737	92·7
Nuclei + 100 μg. puromycin	Methionine-M-^{14}C	318 $\pm$ 0·01	215	120	135	95·8

*Proteins plus ribosomes extractable in 0·01 *M* tris buffer (*p*H 7·6).

†Methyl-lysine separated from methionine on Dowex-50, eluted with the basic amino acids, and counted by scintillation.

Effects of acetylated histones on RNA synthesis

Is there a connection between acetylation of the histones and their role in the regulation of RNA synthesis ?

Evidence that histones can inhibit RNA synthesis in the nucleus was presented in 1961 (Ref. 3) and it was later shown that selective removal of the histones leads to increased rates of "messenger" RNA synthesis[6]. These findings, together with work on isolated enzyme systems[7-11], have led to the view that histone-DNA complexes are unable to serve as "primers" for RNA synthesis[7].

This view does not allow any obvious or easy mechanism for reversing the histone inhibition, except by removal of the histones. However, the experiments now to be described indicate that acetylation of the histones can lower their effectiveness as inhibitors of the RNA polymerase reaction, and suggest a dynamic and reversible mechanism for "activation" as well as "repression" of RNA synthesis.

Acetylation of isolated histones. To test the effects of acetylation on the inhibitory action of histones, we selected the arginine-rich histone fraction and employed limited acetylation by chemical methods.

Effects of acetylated histones on RNA polymerase reactions. Calf thymus nuclei are a good source of an "aggregate-enzyme" preparation which can incorporate nucleoside triphosphates into RNA[8,39]. The reaction can be followed by measuring the incorporation of ATP-8-^{14}C or UTP-^{14}C into an acid-insoluble RNAase-sensitive product[8,40]. The formation of RNA is blocked by DNAase treatment, by actinomycin D, and by the addition of thymus histones[8,40].

The effects of adding arginine-rich histones on RNA synthesis by the "aggregate enzyme" are shown in Table 4. Inhibition, though variable from one experiment to another, usually ranges from 60 to 75%. On the other hand, when histones of varying degrees of acetylation are added to this system, the inhibition is markedly reduced. Even small amounts of acetylation, which hardly affect the electrophoretic mobility of the protein (Table 4) or its ability to combine with DNA (Fig. 3), can lower the inhibition from 66 to 21% of the control value. More highly acetylated histones, although still basic, hardly inhibit at all (Table 4).

Similar results have been obtained with RNA polymerases from *Esch. coli* and *Azotobacter vinelandii* (We are greatly indebted to Dr Edward Reich of The Rockefeller Institute for the gift of the *Esch. coli* enzyme, and to Dr J. S. Krakow of New York University for supplying the Azotobacter enzyme). The *Esch. coli* results are included in Table 4; as in the case of the thymus enzyme, the acetylated histones did not inhibit RNA synthesis as effectively as did the parent proteins.

The failure of acetylated histones to block the RNA polymerase reaction is not due to their inability to combine with DNA. On the contrary, these proteins very effectively protect DNA from thermal denaturation. In Fig. 3

TABLE 4—EFFECTS OF THYMUS HISTONES AND ACETYLATED HISTONES ON RNA POLYMERASE ACTIVITY

Enzyme source	Histone added	Histone mobility* mm./hr	^{14}C-ATP uptake into RNA† c.p.m.	Inhibition %
Calf thymus	Control—no histone added	..	68·6	..
	Arginine-rich histone (control for acetylation)	..	22·2	67·6
	Arginine-rich histone (untreated)	63	27·6	60
	Acetylated histone I‡	59	49·8	27·4
	Acetylated histone II	46	60·5	11·8
	Acetylated histone III	40	68·7	0
Esch. coli	Control—no histone added	..	550	..
	Arginine-rich histone (control for acetylation)	..	317	42·4
	Arginine-rich histone (untreated)	63	288	47·6
	Acetylated histone II	46	429	22·0

*Electrophoretic mobility on cellulose polyacetate (Sepraphore III); 0·1 *M* acetate (*p*H 4·0); 6 V./cm.

†Average of 5 separate experiments for thymus RNA polymerase, and of 2 experiments for *Esch. coli* polymerase.

‡Acetylated histones numbered in order of increasing acetylation, using 0·032, 0.32 and 3·2 meq. acetic anhydride per 100 mg. arginine-rich histone.

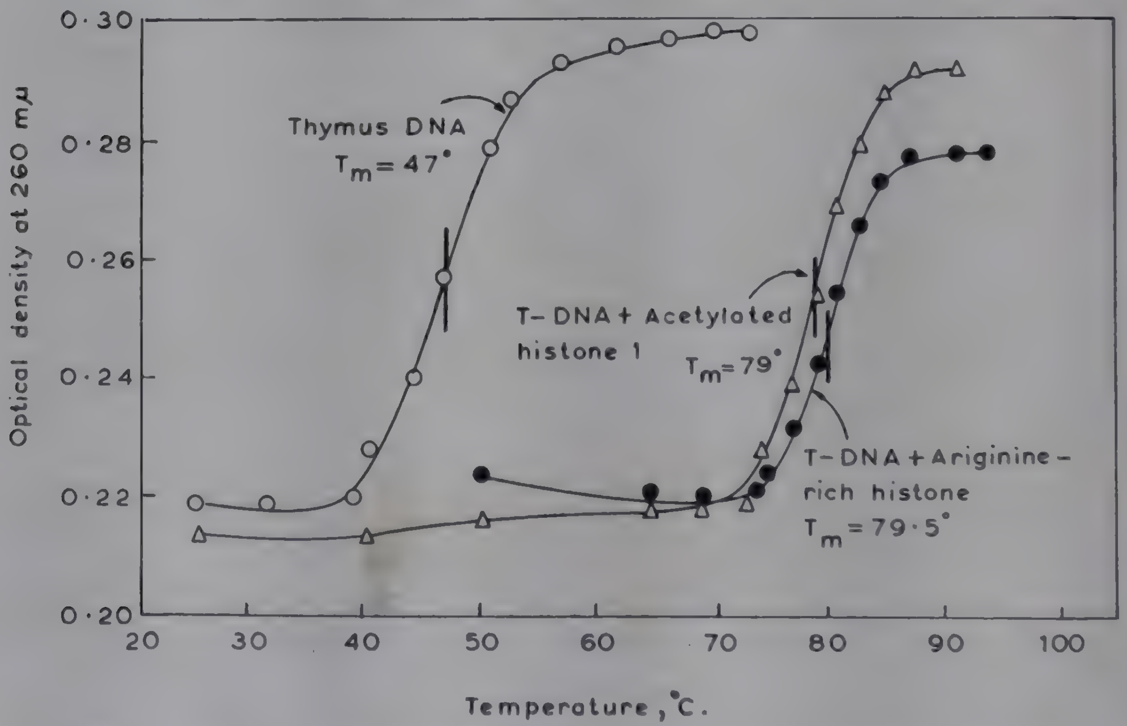

FIG. 3—THERMAL DENATURATION CURVES FOR CALF THYMUS DNA AND FOR COMPLEXES OF DNA WITH THE ARGININE-RICH HISTONE FRACTION AND WITH A WEAKLY ACETYLATED ARGININE-RICH HISTONE (0·032 meq. ACETIC ANHYDRIDE PER 100 mg. HISTONE): DNA and histone were dissolved in 1 *M* NaCl at 20 g./ml. and dialysed against 5×10^{-4} *M* tris (HCl) buffer (*p*H 7·4). Optical density at 260 mμ is plotted against temperature, and Ts are indicated by the dark lines crossing the curves

the melting curves are shown for DNA, for the DNA-arginine-rich histone complex (66% inhibition of RNA synthesis), and for the DNA-acetylated histone complex (21% inhibition of RNA synthesis). It should be noted that the denaturation studies were carried out at the same *p*H value (7·4) as were the RNA polymerase assays.

Acetylated histones are also able to combine effectively with nuclei from which the histones have been previously extracted with ethanolic HCl (Mirsky, W. E., unpublished data).

The results make it clear that complexes of DNA with acetylated histones can be formed, but under these conditions, 'primer' activity for RNA synthesis is not lost. It may be suggested that specificity in DNA-histone binding, alterable by acetylation of the histone, can influence the rate of RNA synthesis. This would allow means of switching-on or -off RNA synthesis at different times, and at different loci of the chromosomes. Although the evidence supporting this view is, at best,tentative (because the acetylated histones tested were acetylated after isolation), it should be recalled that histone acetylation does occur in the nucleus and that its time course resembles that for RNA synthesis.

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